United States Patent Application *20040023909*
Kind Code **A1**
Roy-Chowdhury, Jayanta ; et al. **February 5, 2004**

Process for preventing or reducing undesirable immunological effects to infectious agents in subjects

Abstract

Novel immune modulating processes are provided in which the imunological state of a subject including mature subjects, mammals and humans, are down regulated in a selective manner, and as a subset in a dominant manner. The novel immunological state termed SIDR for selective immune down regulation is usefully applied to the immunological modulation or regulation of gene delivery components, artificially expressed genes, gene delivery systems and expression products of artificially introduced genes by such delivery systems, and infectious agents. SIDR is also useful when combined with other immune modulating treatments such as general immune suppression and anti-apoptosis. SIDR may also be used to selectively down regulate the immune response system of a subject to a wide variety of noncellular immunogenic components and to native antigens. Other processes for producing immune suppression by administering macromolecules or compounds to a subject so as to obtain or effect SIDR are also provided. Kits for carrying out the novel processes are also provided.

Inventors: **Roy-Chowdhury, Jayanta**; *(New Rochelle, NY)* ; **Ilan, Yaron**; *(Bronx, NY)* ; **Rabbani, Elazar**; *(New York, NY)* ; **Engelhardt, Dean L.**; *(New York, NY)*

Correspondence Name and Address:
HUNTON & WILLIAMS
INTELLECTUAL PROPERTY DEPARTMENT
1900 K STREET, N.W.
SUITE 1200
WASHINGTON
DC
20006-1109
US

Assignee Name and Adress: **Enzo Therapeutics, Inc**

Serial No.: **377603**
Series Code: **10**

h f g e cg h e ec ec

Filed: **March 4, 2003**

U.S. Current Class:	**514/44**; 424/145.1; 424/93.2
U.S. Class at Publication:	**514/44**; 424/93.2; 424/145.1
Intern'l Class:	A61K 039/395; A61K 048/00

Claims

What is claimed is:

1. A process for producing selective immune down regulation in an adult subject to a gene delivery component comprising introducing into said adult subject a reagent or a combination of reagents capable of producing selective immune down regulation.

2. The process of claim 1, wherein said selective immune down regulation is dominant.

3. The process of claim 1, wherein said gene delivery component is viral.

4. The process of claim 2, wherein said gene delivery component is viral.

5. The process of claim 4, wherein said viral component comprises adenovirus.

6. The process of claim 1, wherein said gene delivery component is non-viral.

7. The process of claim 6, wherein said non-viral component comprises a non-viral protein or ligand.

8. The process of claim 1, wherein said reagent or combination of reagents are introduced continuously.

9. The process of claim 1, wherein said reagent or combination of reagents are introduced in a series of separate administrations.

10. The process of claim 1, further comprising comprising administering to said subject one or more anti-apoptotic agents.

11. The process of claim 11, wherein said one or more anti-apoptotic agents are selected from the group consisting of physiologic inhibitors, viral genes and pharmacological agents, or a combination of any of the foregoing.

12. The process of claim 11, wherein said anti-apoptotic agent comprises an antibody directed against an apoptotic factor or an antibody directed against a cytokine.

13. The process of claim 1, wherein said adult subject is a mammal.

14. The process of claim 13, wherein said mammal is human.

15. A kit useful for producing selective immune down regulation in an adult subject to a gene delivery component, said kit comprising in packaged combination or containers a reagent or reagents capable of producing selective immune down regulation, and buffers and instructions therefor.

h f g e cg h e ec ec

16. A process for producing selective immune down regulation in an adult subject to an artificially expressed gene within said adult subject, the process comprising introducing into said adult subject a reagent or a combination of reagents capable of producing selective immune down regulation, said reagent or combination of reagents comprising a product or product fragment expressed from said gene.

17. The process of claim 16, wherein said selective immune down regulation is dominant or said reagent or combination of reagents are capable of producing dominant immune down regulation.

18. The process of claim 16, wherein said gene is native or non-native.

19. The process of claim 16, wherein said gene is viral.

20. The process of claim 17, wherein said gene is viral.

21. The process of claim 20, wherein said viral gene comprises adenovirus.

22. The process of claim 16, wherein said gene is non-viral.

23. The process of claim 16, further comprising comprising administering to said subject one or more anti-apoptotic agents.

24. The process of claim 23, wherein said one or more anti-apoptotic agents are selected from the group consisting of physiologic inhibitors, viral genes and pharmacological agents, or a combination of any of the foregoing.

25. The process of claim 23, wherein said anti-apoptotic agent comprises an antibody directed against an apoptotic factor or an antibody directed against a cytokine.

26. The process of claim 16, wherein said subject is a mammal.

27. The process of claim 26, wherein said mammal is a human.

28. A kit useful for producing selective immune down regulation in an adult subject to an artificially expressed gene, said kit comprising in packaged combination or containers a reagent or reagents capable of producing selective immune down regulation in an adult subject, and buffers and instructions therefor.

29. A process for producing selective immune down regulation in an adult subject to a gene delivery system and to a product from expression of an artificially introduced gene by said delivery system in said adult subject, said process comprising introducing to said adult subject a reagent or a combination of reagents capable of producing selective immune down regulation, said reagent or reagents comprising a component or components of said delivery system and a product or product fragment expressed from said gene.

30. The process of claim 29, wherein said selective immune down regulation is dominant or said reagent or combination of reagents are capable of producing dominant immune down regulation.

31. The process of claim 29, wherein said gene is native or non-native.

32. The process of claim 29, wherein said gene delivery system or component, or said expressed gene,

h f g e cg h e ec ec

or both, are viral.

33. The process of claim 30, wherein said gene delivery system or component, or said expressed gene, or both, are viral.

34. The process of claim 33, wherein said viral system or component, or said expressed viral gene, or both, comprise adenovirus.

35. The process of claim 29, wherein said gene delivery system or component, or said expressed gene, or both, are non-viral.

36. The process of claim 29, further comprising administering to said subject one or more anti-apoptotic agents.

37. The process of claim 36, wherein said anti-apoptotic agents are selected from the group consisting of physiologic inhibitors, viral genes and pharmacological agents, or a combination of any of the foregoing.

38. The process of claim 36, wherein said anti-apoptotic agent comprises an antibody directed against an apoptotic factor or an antibody directed against a cytokine.

39. The process of claim 29, wherein said adult subject is a mammal.

40. The process of claim 39, wherein said mammal is a human.

41. A kit useful for producing selective immune down regulation in an adult subject to gene delivery or to expression of an artificially introduced gene in said adult subject, said kit comprising in packaged combination or containers (i) a reagent or a combination of reagents capable of producing selective immune down regulation, and optionally, (ii) one or more anti-apoptotic agents, and buffers and instructions therefor.

42. A process for producing selective immune down regulation in a subject to an infectious agent comprising introducing to said subject a reagent or a combination of reagents capable of producing selective immune down regulation and comprising a component or components or fragments thereof of said infectious agent.

43. The process of claim 42, wherein said infectious agent is selected from the group consisting of bacteria, viruses and fungi, or a combination of any of the foregoing.

44. The process of claim 43, wherein said viral infectious agent is selected from the group consisting of HBV, HCV, HIV-1, HIV-2, HTLV-1, CMV, EBV and HSV, or a combination of any of the foregoing.

45. The process of claim 42, wherein said infectious agent component or components or fragments thereof are contained within a cell matrix of said subject, or are complexed with a cell receptor or antibodies of said subject, or any conjugates derived from the foregoing.

46. The process of claim 42, wherein said selective immune down regulation is dominant or said reagent or combination of reagents are capable of producing dominant immune down regulation.

47. The process of claim 46, wherein said dominant immune down regulation is effected by administering at least one component or a fragment of said infectious agent or a cell containing a

h f g e cg h e ec ec

component or fragment of said infectious agent.

48. The process of claim 43, further comprising treating said subject with an effective amount of a compound selected from the group consisting of antiviral compounds, antibacterial compounds and antifungal compounds, or a combination of any of the foregoing.

49. The process of claim 48, wherein said antiviral compounds comprise a member selected from the group consisting of chemotherapeutic agents, enzyme inhibitors, and interferons, or a combination of any of the foregoing.

50. The process of claim 42, further comprising comprising administering to said subject one or more anti-apoptotic agents.

51. The process of claim 50, wherein said one or more apoptotic agents are selected from the group consisting of physiologic inhibitors, viral genes and pharmacological agents, or a combination of any of the foregoing.

52. The process of claim 50, wherein said anti-apoptotic agent comprises an antibody directed against an apoptotic factor or an antibody directed against a cytokine.

53. The process of claim 42 or 51, further comprising exposing said subject to at least one other immune modulating treatment selected from immune suppression and selective immune down regulation.

54. The process of claim 42, wherein said subject is a mammal.

55. The process of claim 54, wherein said mammal is a human.

56. A kit useful for producing selective immune down regulation in a subject to an infectious agent comprising in packaged combination or containers (i) a reagent or a combination of reagents capable of producing selective immune down regulation, said reagent or combination of reagents comprising a component or components or fragments thereof of said infectious agent, and (ii) buffers and instructions therefor.

57. A process for producing immunological tolerance in a subject to a gene delivery component or to an artificially expressed gene in said subject, or to both, said process comprising subjecting said subject to more than one immune modulating treatment, at least one of which treatment is selective immune down regulation and at least one other treatment is selected from the group consisting of general immune suppression, anti-apoptosis and selective immune down regulation.

58. The process of claim 57, wherein said at least two immune modulating treatments are selected from the groups consisting of: selective immune down regulation and general immune suppression; selective immune down regulation and anti-apoptosis; and selective immune down regulation, immune suppression and anti-apoptosis.

59. The process of claim 57, wherein the subject is exposed to said at least two immune modulating treatments prior to administration of said gene delivery component or expression of said artificially expressed gene.

60. The process of claim 57, wherein the subject is exposed to said at least two immune modulating treatments after administration of said gene delivery component or expression of said artificially expressed gene.

h f g e cg h e ec ec

61. The process of claim 57, wherein the subject is exposed to said at least two immune modulating treatments at substantially the same time as said gene delivery component is administered or said gene is artificially expressed.

62. The process of claim 57, wherein the subject is simultaneously exposed to said at least two immune modulating treatments.

63. The process of claim 57, wherein the subject is exposed to said at least two immune modulating treatments at different times.

64. The process of claim 57, wherein said selective immune down regulation is dominant.

65. The process of claim 57, wherein said gene delivery component, or said expressed gene, or both, are viral.

66. The process of claim 65, wherein said viral component, or said expressed viral gene, or both, comprise adenovirus.

67. The process of claim 57, wherein said gene delivery component, or said expressed gene, or both, are non-viral.

68. The process of claim 57, wherein said immune suppression is effected by administering an effective amount of an immunosuppressive compound to said subject.

69. The process of claim 68, wherein said immunosuppressive compound is selected from the group consisting of a corticosteroid, a cytotoxic drug, cyclosporine, and an antilymphocyte antibody, or a combination of any of the foregoing.

70. The process of claim 69, wherein said antilymphocyte antibody comprises a polyclonal antibody or a monoclonal antibody.

71. The process of claim 57, wherein said anti-apoptosis treatment is carried out by administering to said subject one or more anti-apoptotic agents selected from the group consisting of physiologic inhibitors, viral genes and pharmacological agents, or a combination of any of the foregoing.

72. The process of claim 57, wherein said subject is a mammal.

73. The process of claim 72, wherein said mammal is a human.

74. The process of claim 57, wherein both a gene delivery component is introduced into said subject and a gene is artificially expressed in said subject.

75. A kit useful for producing selective immune down regulation in a subject to a gene delivery component or to an artificially expressed gene, the kit comprising in packaged combination or containers reagents or a combination of reagents capable of producing selective immune down regulation, and at least one other means for generating general immune suppression, or anti-apoptotic effects in said subject, or both, and buffers and instructions therefor.

76. A process for producing selective immune down regulation in a subject to a noncellular immunogenic component capable of biological function or interfering with biological function in said

h f g e cg h e ec ec

subject, said process comprising introducing into said subject a reagent or combination of reagents capable of producing selective immune down regulation.

77. The process of claim 76, wherein said selective immune down regulation is dominant or said reagent or combination of reagents are capable of producing dominant immune down regulation.

78. The process of claim 76, wherein said noncellular immunogenic component is selected from the group consisting of an antibody, an antibody/antigen complex, an antibody/antigen cell matrix, an enzyme, an antitumor protein or protein inhibitor, a receptor, a hormone, a ligand, an effector and an inducer, or a combination of any of the foregoing.

79. The process of claim 78, wherein said antibody or said antibody in said antibody/antigen complex or antibody/antigen cell matrix is polyclonal or monoclonal.

80. The process of claims 78 or 79, wherein said antibody is directed to one or more epitopes on an immune cell.

81. The process of claim 80, wherein said epitope is selected from the group consisting of CD2, CD4, CD8, CTLA41g, OTK, anti-Th, or a combination of any of the foregoing.

82. The process of claims 78 or 79, wherein said antibody is directed to a member selected from the group consisting of an apoptotic factor, a lymphokine, a cytokinin, and a histocompatibility factor, or a combination thereof.

83. The process of claim 82, wherein said histocompatibility factor is selected from MHC Class I and MHC Class II.

84. The process of claim 78, wherein said enzyme comprises a metabolic enzyme involved in the conversion, consumption or degradation of a metabolic product or intermediate.

85. The process of claim 84, wherein said metabolic enzyme is selected from the group consisting of L-asparaginase, superoxide dismutase, bilirubin oxidase, and adenosine deaminase, or a combination of any of the foregoing.

86. A kit useful for producing selective immune down regulation in a subject to a noncellular immunogenic component capable of eliciting a biological function, said kit comprising in packaged combination or containers a reagent or combination of reagents capable of producing selective immune down regulation.

87. The process of any of claims 1, 16, 42 or 57, wherein said selective immune down regulation is effected or obtained by means of oral tolerization.

88. The process of any of claims 1, 16, 42 or 57, wherein said selective immune down regulation is effected through a selective immune suppressive.

89. The process of claim 88, wherein said selective immune down regulation is dominant.

90. The process of claim 88, wherein said selective immune suppressive comprises one or more members selected from an immune suppressor, an antibody to a T cell, an immune suppressive drug, and a cytokine, or a combination of any of the foregoing.

h f g e cg h e ec ec

91. The process of claim 90, wherein said antibody to a T cell is selected from the group consisting of anti-CD4, anti-CD8 and OTK, or a combination of any of the foregoing.

92. A process for producing selective immune down regulation in a subject to a native antigen or group of native antigens comprising subjecting said subject to at least two separate immune modulating treatments at least one of which comprises oral tolerization.

93. The process of claim 92, wherein said native antigen or group of native antigens are derived from the subject's cell or tissue, or fragments thereof, or from the subject's cell or tissue or fragments complexed with antibodies, or from partial digests of any of the foregoing.

94. The process of claim 93, wherein said antigen or group of antigens are selected from the group consisting of collagen, islet cell, liver cell, kidney cell, heart cell, pancreatic cells, spleen cell, and nucleic acid, or a combination of any of the foregoing.

95. The process of claim 92, wherein said antigen or group of antigens comprise a cell, tissue, organ, or components or fragments thereof, transplanted from a donor.

96. The process of claim 95, wherein said donor has been treated with the subject's cells, or tissues or fragments or conjugates to obtain selective immune down regulation prior to transplantation of said cell, tissue organ, or components or fragments thereof to said subject.

97. The process of claims 95 or 96, wherein said donor's cell, tissue, organ, or components or fragments thereof are derived or taken from skin.

98. The process of claims 95 or 96, wherein said donor's cell or tissue comprises bone marrow.

99. The process of claim 92, wherein the second treatment is selected from the group consisting of selective immune down regulation, immune suppression, and anti-apoptosis.

100. The process of claim 92, wherein said at least two separate immune modulating treatments both or all comprises selective immune down regulation.

101. The process of claim 92, further comprising administering at least one cytokine to said subject.

102. The process of claim 92, wherein said at least two separate immune modulating treatments are given repeatedly in a single dosage period or in a series of dosage periods.

103. The process of claim 92, wherein said at least two separate immune modulating treatments are given separately or concurrently.

104. The process of claim 92, wherein said subject is sensitive or naive to said antigen or group of antigens.

105. The process of claim 92, wherein said subject is a mammal.

106. The process of claim 105, wherein said mammal is a human.

107. A process for producing immune suppression in a subject comprising administering macromolecules or compounds to said subject, said macromolecules or compounds being immunogenic or being capable of providing immune suppression, wherein said subject was treated to obtain selective

immune down regulation to said macromolecules or compounds, permitting thereby repeated use of said macromolecules or compounds with substantially little or no immune response.

108. A process for transiently producing selective immune down regulation in a subject to a specific antigen comprising transferring non-native cells from a donor to said subject, wherein said donor that has dominant selective immune down regulation.

109. The process of claim 108, wherein said subject is immunosuppressed prior to or during said transferring step.

110. The process of claim 108, wherein said subject is immunosuppressed prior to and during said transferring step.

111. A process for producing selective immune down regulation in a subject to an antigen or group of antigens comprising introducing into said subject non-native compounds or non-native immunological reagents capable of producing immune suppression in said subject, wherein prior to or during or prior to and during said introduction step said subject is exposed to said antigen or group of antigens, and wherein said subject has been subjected to selective immune down regulation to said non-native compounds or non-native immunological reagents.

112. The process of claim 111, wherein said antigen or group of antigens are native to said subject.

113. The process of claim 111, wherein said antigen or group of antigens are transplanted from a donor to said subject.

114. The process of claim 111, wherein selective immune down regulation comprises antibodies to T cells.

115. The process of claim 114, wherein said antibodies are directed against CD4, CD8 and OTK, or a combination of any of the foregoing.

116. The process of claim 111, further comprising administering at least one cytokine to said subject.

117. A transplantation process comprising introducing into a recipient subject (i) a donor liver or cells from a donor liver, and (ii) cells, tissue or organs from said donor, wherein said transplanted donor liver or donor liver cells inhibit rejection of said donor cell, tissue or organ by said recipient.

118. The process of claim 117, wherein said cells from the donor liver comprise immune cells.

119. The process of claim 117, wherein said cells from the donor liver comprise dendritic cells.

120. The process of claim 117, wherein said cells, tissues or organ from said donor to be transplanted are selected from the group consisting of bone marrow, kidney, heart, lung, pancreas, islet cells, skin, bone, or cells or tissues derived from any of the foregoing.

121. A transplantation process comprising the steps of: establishing selective immune down regulation in a recipient subject to the antigens of a donor; and introducing into said recipient subject cells, tissue, or organs, or components thereof from said donor.

122. The process of claim 121, wherein at least one immune modulating treatment has been administered to said recipient subject or said donor or both.

h f g e cg h e ec ec

123. A transplantation process comprising transplanting cells, tissue or organs from a donor to a recipient subject, wherein said recipient subject has been subjected to at least two independent immune modulating treatments, at least one of which comprises selective immune down regulation.

124. The process of claim 123, wherein said cells, tissue or organs from the donor comprise bone barrow.

125. A process of inducing tolerance in a first subject comprising transferring cells from a second subject to said first subject, wherein selected immune down regulation has been established in said second subject by the transfer of immune cells.

Description

FIELD OF THE INVENTION

[0001] This invention relates to the field of immunology and to novel processes for the modulation of immune responses including particularly the down regulation of the immune response system using procedures or combinations of procedures for producing and applying a new and unexpected immune modulation termed selective immune down regulation.

[0002] All patents, patent applications, patent publications, scientific articles, and the like, cited or identified in this application are hereby incorporated by reference in their entirety in order to describe more fully the state of the art to which the present invention pertains.

BACKGROUND OF THE INVENTION

[0003] While immunological responses are essential in animals and man, certain undesirable consequences of these responses may occur and often do. A partial list of examples of such undesirable responses include autoimmune disease, serum sickness, rejection of cell, tissue and organ transplants, rejection of desirable non-native components, immune complex-based destruction of certain tissues, tissue and cell destruction based on the induction of apoptosis or cell death, certain actions of antiidiotypic antibodies and certain anaphylactic responses.

[0004] One aspect of this problem revolves around the unwanted immune response to the production or presentation of non-native compounds, proteins or other antigenic materials (not found in the subject in which they have been placed) or proteins or other antigenic materials which are native constituents of the subject, but for some reason have been rendered immunogenic. In the latter instance, these specific proteins or other antigenic materials could have been rendered immunogenic for a number of reasons. They may have been modified to express a new antigenic determinant. An aberrant site of expression may have developed or even the amount of these proteins may have been altered rendering them immunogenic.

[0005] Unwanted host immune response can occur in the course of gene therapy. The immune response can be directed against antigens present in the vector and/or the products of the transferred genes. In general, the undesired production or presentation of antigens can result from the use of any viral or non-viral gene delivery system. Such an immune response can shorten the duration of expression of transgenes and can substantially reduce or inhibit a repeat of the transduction to reinstate these genes, thus posing a major hurdle to long-term gene therapy.

h f g e cg h e ec ec

[0006] While dependence and reliance on immune response is both necessary and required, strategies to prevent or to overcome the undesirable consequences of certain immunological responses, are limited, and often ineffective. In general, immunological suppressive methods and procedures lead to overall suppression of the immune system. Maintaining a prolonged state of immunological suppression by overall suppression of the immune system is not desirable if it can be avoided.

[0007] In contrast to general immunosuppression, tolerance to specific antigens (such as adenovirus particles) can be induced if the antigen is injected into a neonate or into a fetus (Takahashi et al., J Biol Chem:271:26536-26534 (1996)); (Hagstrom, et al., Proc Natl Acad Sci 93:3056-61 (1996)). However, this procedure has a major limitation. It is effective only in the fetus or during the first few days after birth (not an adult).

[0008] Another tolerization protocol involves the direct injection of a soluble antigen into the functional thymus. (Ilan, et al., J Clin Invest, 98:2640-2647 (1996). This modality of tolerization is not applicable to adult subjects since such subjects lack an active thymus (not an adult).

[0009] Certain infections could lead to an autoimmune response in which both infected and/or uninfected cells are subjected to an undesirable immune response. Examples of such responses are hepatitis B infection, HIV infection and rheumatic fever. Because immune response complications are intermingled with the element of infection, there is currently no effective cure or management strategy for these diseases.

[0010] Furthermore, the use of many non-native compounds (adenovirus, for example) that are immunogenic in a subject, is also limited or inhibited due to the immunological response of the subject to these compounds and reagents.

[0011] Immunological modulation is an artificially induced variation in a subject's immune system in response to the introduction of reagents, procedures and processes. Such modulation could be based on an immune response that is humoral or cellular or both which in turn occurs in response to a non-native compound. Immunological modulation could be used to suppress an immunological response broadly or narrowly.

SUMMARY OF THE INVENTION

[0012] Novel processes and kits for producing selective immune down regulation (SIDR) and immune suppression in subjects are provided by this invention. Among the novel processes are those for producing SIDR in an adult subject to gene delivery components. SIDR is produced in such a subject by introducing a reagent or a combination of reagents capable of producing SIDR.

[0013] Other novel processes and kits for producing SIDR include those in which an adult subject is challenged by an artificially expressed gene. In such other processes, the SIDR is produced by introducing into the subject a reagent or combination of reagents capable of producing SIDR in which a product or a product fragment artificially expressed from the gene in question is formulated into such a reagent or combination of reagents.

[0014] This invention additionally provides novel processes for producing SIDR in an adult subject that is directed to both a gene delivery system and to an expression product from an artificially introduced gene by such delivery system. A reagent or combination of reagents capable of producing SIDR are introduced into the adult subject, the reagent or combination comprising a component or components from the gene delivery system and a product or product fragment expressed from the artificially

h f g e cg h e ec ec

introduced gene. A kit useful for carrying out such novel processes is also provided by this invention.

[0015] Another unique aspect of this invention concerns processes for producing SIDR in any subject to a wide variety of infectious agents, including bacteria, viruses and fungi. In this aspect, a reagent or combination of reagents are introduced into the subject wherein the reagent or combination of reagents are capable of producing SIDR and they comprise some part of the infectious agent in question, be it a component or components or a fragment or fragments. A kit is also provided in which the SIDR producing reagent or reagent combination is formulated as an element for carrying out this process.

[0016] Another important feature of this invention relates to processes and kits for producing immunological tolerance in any subject, e.g., a mammal such as a human. In this feature, the subject is treated, exposed or subjected to more than one immune modulating treatments or regimen--at least one of which must be SIDR. The other treatment can also be SIDR, or it can take the form of general immune suppression or anti-apoptosis.

[0017] This invention is also related to novel processes for producing SIDR in any subject to a widely diverse range of noncellular components capable of biological function or interfering with biological function in any subject. In these processes, a reagent or a combination of reagents having SIDR capability are introduced into a subject. The noncellular components are numerous and diverse covering such things as antibodies, antibody/antigen complexes, antibody/antigen cell matrices, enzymes, antitumor proteins, protein inhibitors, receptors, hormones, ligands, effectors, inducers and combinations of the like. Reagents or combinations of reagents can be usefully formulated into kits for carrying out such novel processes as just briefly described.

[0018] Another feature of this invention relates to processes for producing SIDR in any subject to a native antigen or a group of native antigens. To so produce SIDR, a subject is given or exposed, treated or subjected to two or more separate and distinct immune modulating treatments, one of which must be oral tolerization as described in further detail below.

[0019] Other novel processes are provided in this invention. One such process concerns immune suppression production in a subject by administering macromolecules or compounds to the subject. The macromolecules or compounds are immunogenic themselves, or they possess the capability of providing immune suppression to the subject. In this novel process, the subject is treated to obtain a SIDR state to the macromolecules or compounds. By so doing, repeated use of these macromolecules or compounds can be undertaken with substantially little or greatly reduced immune response. In some instances, the immune response may be for all intents and purposes shut down with respect to the macromolecules or compounds.

[0020] Another novel process is provided where a transient SIDR state is obtained in a subject by transferring non-native cells from a donor having dominant selective immune down regulation to the subject under study.

[0021] In a further aspect of this invention processes are provided for producing SIDR in any subject to any antigen or group of antigens, including native antigens and those other antigens that have been transplanted from a donor to the subject under study. In this instance, non-native compounds or non-native immunological reagents capable of producing immune suppression are introduced into the subject. Either prior to or during the introduction, or even from before and up to and including the introduction the subject--who has been subjected to SIDR--is exposed or challenged by the antigen or group of antigens.

[0022] More particular details and embodiments of the invention are described more fully below in the

detailed description and preferred embodiment sections of this application that follow.

BRIEF DESCRIPTION OF THE FIGURES

[0023] FIG. 1 demonstrates .beta.-galactosidase expression in liver specimens from orally tolerized rats (group B) and the control rats (Group C2) after the second injection of the recombinant virus.

[0024] FIG. 2 shows PCR gel results from the detection of the presence of human BUGT.sub.1 DNA in rat livers after the second injection of the recombinant virus.

[0025] FIG. 3 is a Western blot analysis of expression of human BUGT.sub.1 after the second injection of the recombinant virus.

[0026] FIG. 4 shows the results of the effect of tolerization upon bilirubin levels after the second injection of the recombinant virus.

[0027] FIG. 5 depicts the anti-adenovirus antibody levels in group A tolerized (solid bars) and group C control (open bars) rats after the first and second injection of the recombinant virus.

[0028] FIG. 6 are micrographs of liver biopsies taken taken 24-72 hours after the second injection showing minimal lymphocytic infiltration in tolerized rats (A) and severe inflammation in the control rats (B).

[0029] FIG. 7 are PCR gel results from the detection of the presence of human BUGT.sub.1 DNA in rat livers after the second injection of the recombinant virus.

[0030] FIG. 8 is a Western blot analysis of expression of human BUGT.sub.1 after the second injection of the recombinant virus.

[0031] FIG. 9 is a graph showing the effect of tolerization upon bilirubin levels after the second injection of the recombinant virus.

[0032] FIG. 10 is a graph showing serum bilirubin levels after adoptive transfer.

[0033] FIG. 11 is a color micrograph for .beta.-galactosidase expression in liver specimens from rabbits after first injection.

[0034] FIG. 12 is also a color micrograph for .beta.-galactosidase expression in liver specimens from rabbits three weeks after first injection.

[0035] FIG. 13 is also a color micrograph for .beta.-galactosidase expression in liver specimens from orally tolerized rabbits after second injection.

[0036] FIG. 14 is also a color micrograph of .beta.-galactosidase expression in liver specimens from non-tolerized control rabbits after second injection.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present invention provides, among other things, a new immune modulating process in which the immune response system of a subject can be specifically down regulated. This novel approach to immune modulation in which undesirable or deleterious immune reactions are specifically suppressed in

h f g e cg h e ec ec

a subject has been termed selective immune down regulation or SIDR.

[0038] The present invention provides processes, kits and compositions in the form of reagents for producing this unique SIDR condition. In attaining SIDR, the present invention relies on immunomodulation procedures to facilitate the introduction or incorporation of novel biologically functional non-native compounds or non-native cellular material in a subject who can be a mammal, including a human, or an adult human. Another aspect of this invention is to uncouple the immunological response to infectious agents from the propagative aspects of said infectious agents through immunological modulation. It is a further aspect and principle of this invention that a single immunological suppression approach in and of itself cannot lead to effective inhibition of the subject's immunological system. It has now been found, however, that a combination of independent and separate immunosuppression approaches in such a subject could approach or attain an effective inhibition of the immune system. In such an inhibited state, and in the presence of an immunologic stimulus (for example the introduction of an antigen or a group of antigens), the immune system of the subject will no longer remain naive with respect to said antigen or antigens, but will proceed to launch both an immune response, as well as an immune tolerance. This immunological duality--immune responsiveness and immune tolerance--allows the subject to be competent against all antigens other than the selected antigen for which tolerance is desirable. In applying combined immunosuppressive procedures and treatments that would sufficiently inhibit the immune response while allowing the development of immune tolerance, the subject will develop SIDR.

[0039] In some instances a given immune modulation that can train the immune system in itself may not produce the desired immunological or biological effects sufficiently to produce optimal results. Thus, another aspect of this invention is to improve such a process by combining at least one oral tolerization procedure with any other immune modulation procedures which could even include two or more such immune modulating procedures or treatments. Such combinations could take the form of two independent and separate selective immune down regulation procedures both directed to a given specific antigen or antigens. For example, two such selective immune down regulation procedures could comprise oral tolerization and selective immune suppression. Such a procedure could further comprise the use of other immune modulating procedures such as immunosuppressive drugs, appropriate cytokines, adjuvants, conjugates, or combinations thereof.

[0040] As used herein, "selective immune down-regulation" (SIDR) is an immunological state in a subject or biological system in which the subject maintains tolerance (prevention or suppression of a specific immune response) to a particular antigen or set of antigens (or other immunological determinant (s)) while at the same time maintaining immunological competence against other antigens, or other classes of antigens or immunological determinants. Furthermore, in such a state, SIDR is capable of being maintained in the subject after immunological processes or modulation that has led to the SIDR state has ceased or terminated.

[0041] As used herein, the term "dominant" as employed with immune down regulation or DIDR refers to a particular form of SIDR. If the SIDR state can be transferred and manifested as a dominant state in the new subject, then such a state is defined as a dominant immune down-regulation (DIDR) state.

[0042] As used herein, the term "general immune suppression or suppressives" (GIS) refers to immune modulating reagents or procedures which could lead to the prevention of an immune response that is not specific to any particular antigen or set of antigens but rather is indiscriminate, non-specific and general. Such an immune suppression can be maintained in general if the reagents or procedures are themselves maintained. Such reagents or procedures can be administered transiently, repeatedly, or over prolonged duration.

h f g e cg h e ec ec

[0043] Among the novel processes provided by this invention is one for producing selective immune down regulation in an adult subject to a gene delivery component. This novel process comprises introducing into the adult subject a reagent or a combination of reagents capable of producing selective immune down regulation. In a further aspect of the just-described process, the SIDR can be dominant, a term and state defined above. The gene delivery component may take a wide variety of forms, including viral, e.g., adenovirus, and nonviral, e.g., proteins, ligands, or any protein containing or proteinaceous molecule.

[0044] The reagent or combination of reagents that are capable of producing SIDR can comprise some portion or fragment of the gene delivery component. Introduction of the SIDR producing reagent or combination of reagents can be carried out using conventional methodology and procedures that are well known to those skilled in this art. For example, the reagent or combinations can be introduced continuously into the subject, or introduced in a series of separate administrations. The separate administrations may be marked by fixed time intervals or variable time intervals, as the case may be. For a further description of administration and protocols for introducing the reagents or combination of reagents, reference is made to Oral Tolerance: Mechanisms and Applications, H. L. Weiner and L. F. Mayer, eds. (1996) The New York Acadamy of Sciences New York, N.Y., the contents of which are incorporated by reference.

[0045] In another aspect of the aforementioned process, one or more anti-apoptotic agents may be administered to the adult subject. Apoptosis refers to an evolutionarily conserved form of cell suicide and is well described. See, for example, the review article by Wyllie, et al., "Cell Death: The Significance of Apoptosis", International Review of Cytology, Vol. 68. See also, the review articles by Sachs, et al., Blood, 82: 15-21 (1993), Kerr, et al., Br. J. Cancer, 26: 239-257 (1972), and the more recent review article by Thompson, Science, 267: 1456-1462 (1995). All of the foregoing are incorporated by reference. The latter article is particularly useful because it provides several inhibitors of apoptosis on page 1457. These inhibitors or anti-apoptotic agents include a number of physiologic inhibitors, viral genes and pharmacological agents, any or all of which can be used in the instantly described process. A number of textbooks specificially dealing with apoptosis have been published. These include, for example, Tomei's Apoptosis: The Molecular Basis of Cell Death, Cold Spring Harbor Laboratory, Volumes 3 (1991) and 8 (1994); Kroemer's Apoptotis In Immunology, Springer-Verlag, Inc. (1995); and Gregory's Apoptosis And The Immune Response, (1995). The contents of all the foregoing review articles and textbooks are incorporated herein by reference. In a particular aspect of the process, the anti-apoptotic agent can comprise an antibody directed against an apoptotic factor or an antibody directed against a cytokine, including lymphokines.

[0046] To carry out the process and to produce SIDR in an adult subject to a gene delivery component, the present invention contemplates a kit useful for that purpose. The kit comprises in packaged combination or containers a reagent or reagents or particular combinations of reagents capable of producing SIDR to the gene delivery component. These reagents have been described above. Buffers and instructions are other conventional elements of the kit.

[0047] Still another process provided herein produces SIDR in an adult subject to an artificially expressed gene, the gene being expressed within the adult subject. In this process a reagent or combination of reagents are formulated based upon a product or product fragment expressed from the gene of interest. These reagents or the combination of reagents are capable of producing SIDR in an adult subject when so formulated. Further, the reagent or combination of reagents that are introduced into the adult subject can themselves be capable of producing dominant immune down regulation (DIDR).

[0048] In terms of the artificially expressed gene, it may be native or non native to the subject, and it

h f g e cg h e ec ec

will be non viral. A delivery system for such a gene may be viral (e.g., adenovirus) or non-viral. As in the case of other novel processes of this invention, one or more anti-apoptotic agents may be administered to the subject. These agents include any of those selected from physiologic inhibitors, viral genes and pharmacological agents or combinations thereof, including antibodies directed against apoptotic factors or cytokines, as described above and elsewhere. See, for example, Thompson, (1995), supra.

[0049] The present invention also provides a kit useful for producing selective immune down regulation in an adult subject to an artificially expressed gene, said kit comprising in packaged combination or containers a reagent or reagents capable of producing selective immune down regulation in an adult subject, and buffers and instructions therefor.

[0050] Still yet another novel process provided by this invention produces SIDR, a process for producing selective immune down regulation in an adult subject both to a gene delivery system or component thereof and to a product from expression of an artificially introduced gene, the gene having been introduced into the adult subject by the aforementioned gene delivery system. This process comprises introducing into the adult subject a reagent or a combination of reagents capable of producing SIDR. The reagent or reagents or combination of reagents comprise a component or components of the gene delivery system and a product or product fragment expressed from the gene in question. As in the case of other novel processes described above, this process can also be dominant, or the reagents or combination of reagents may be capable of producing DIDR. The nature of the gene (for example, native or non native, viral or non viral), the gene delivery system or component (for example, viral or non viral), the subject (mammal such as human) and the further aspect of administering anti-apoptotic agents (for example, physiologic inhibitors, viral genes and pharmacological agents or antibodies), are all as described above.

[0051] In conjunction with the just described process, the present invention also provides a kit useful for producing SIDR also in an adult subject to the gene delivery or to expression of an artificially introduced gene, in the adult subject. The kit comprises, in packaged combination or containers, (i) a reagent or a combination of reagents capable of producing selective immune down regulation, and, (ii) one or more anti-apoptotic agents, and buffers and instructions therefor. The latter component (ii) represents an optional element of the kit and is designed for a specific preferred aspect of the novel process herein above described.

[0052] A particularly useful application of SIDR involves infectious agents. Here, the novel process produces SIDR in any subject (for example, mammals and humans) to an infectious agent, the latter assuming any number of diverse forms and types, including bacteria, viruses and fungi. Among suitable candidates for viral infectious agents are those selected from the following group: hepatitis B virus (HBV), hepatitis C virus (HCV), human immuno deficiency virus types 1 and 2 (HIV-1 and HIV-2), human T-cell leukemia virus type 1 (HTLV-1), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and herpes simplex virus (HSV). The foregoing list of viral infectious agents is in no way intended to be exhaustive and may very well include others.

[0053] The infectious agent component or components or fragments thereof can be contained within a cell matrix of the subject or they can be complexed with cell receptors or antibodies from the subject or with any conjugates derived from such component, components, fragments, complexes and the like.

[0054] As in the case of other novel processes described herein above, the SIDR in this process can be dominant or the reagent or combination of reagents can possess the capability of producing DIDR. See the above definition for DIDR. DIDR may be effected or obtained by administering to the subject at least one component or a fragment of the infectious agent or even a cell containing a component or a

h f g e cg h e ec ec

fragment of the infectious agent.

[0055] In accordance with a general principle and useful application of SIDR, the SIDR subject can be further treated with a variety of compounds or drugs directed against pathogens. These would include any of the anti-viral compounds, anti-bacterial compounds and anti-fungal compounds. Among the anti-viral compounds are those from the following groups: chemotherapeutic agents, enzyme inhibitors and interferons. The nature, availability and sources, and the administration of all such compounds including the effective amounts necessary to produce desirable effects in a subject are well known in the art and need not be further described herein.

[0056] As in the case of other previously described novel processes, anti-apoptotic agents can be administered to the subject as part of the SIDR producing process. In addition, at least one other immune modulating treatment, e.g., general immune suppression and SIDR, can also be employed in the process.

[0057] For carrying out the just described process against infectious agents the prevent invention also provides a kit useful for producing SIDR in a subject to an infectious agent. In this instance, the kit comprises in packaged combination or containers a reagent or a combination of reagents capable of producing SIDR, and also comprising a component or components or fragments thereof of the infectious agent in question. Buffers and instructions may also be included in this kit.

[0058] One extremely useful application of the present invention is the use of SIDR in combination with other conventional immune modulating treatments. The general immunological suppression procedure can take the form of immunosuppressive drugs, such as cyclosporin or other such drugs, or antibodies to immune cells such as anti-CD4, anti-CD8, OTK, etc. or cytokines or sub-ablative doses of radiation. Immunologic modulation leading to SIDR includes development of specific tolerance by use of general immune suppressors such as CD4 or CD8 antibodies or a combination with other anti-lymphocyte antibodies. While exposing the subject to continuous presence of specific antigenic Immunosuppressive compounds and drugs that are well described in the literature. See for example, Benjamini's Immunology: A Short Course, 2nd Edition, Wiley-Liszt, Inc. (1991), Chapter 19 "Transplantation Immunology," pp. 347-367; and Stites' Basic and Clinical Immunology, 7th Edition, Appleton & Lange, Norwalk, Conn. (1991), Chapter 61 "Immunosuppressive Therapy," pp. 766-779. For more recent textbooks on this subject, please refer to Rich's Clinical Immunology: Principles and Practice, Mosby, St. Louis, Mo., and Samter's Immunologic Diseases, 5th Edition, Little, Brown and Company, Boston. All of the foregoing are incorporated by reference.

[0059] As an aspect of the combination treatment approach, the present invention also provides a process for producing immunological tolerance in a subject to a gene delivery component or to an artificially expressed gene in the subject, or to both the gene delivery component and to a product expressed from the gene. Here, in this novel process, the subject is exposed, treated or otherwise subjected to more than one immune modulating treatment, at least one of which treatment is SIDR. The other treatment or treatments are selected from general immune suppression, anti-apoptosis and SIDR. Thus, in another aspect, two immune modulating treatments can be deployed for purposes of the just described process. For example, SIDR and GIS, SIDR and anti-apoptosis and SIDR, GIS and anti-apoptosis can all be usefully combined as such to produce immunological tolerance in the subject.

[0060] The two or more immune modulating treatments can be administered prior to administration of the gene delivery component or the expression of the artificially expressed gene, or both. Alternatively, the two or more immune modulating treatments can be administered after the gene delivery component or expression of the artificially expressed gene. Alternatively, the two or more treatments can be administered at substantially or approximately the same time as the gene delivery component or the

h f g e cg h e ec ec

expression of the artificially expressed gene. In another aspect, the subject can be exposed simultaneously to the two or more immune modulating treatments or the subject may be exposed to the treatments at different times. The administration including specific reagents or drugs concentrations, mode of administration, monitoring, duration of administration and the like are routinely encountered in the clinical setting and would represent, therefore, information known or available to those skilled in the art.

[0061] As in the case of other novel processes herein before described, the nature of SIDR (for example, dominant or DIDR), the gene delivery component (viral or non viral), the expressed gene and product or fragment expressed therefrom (viral or non viral), the immunosuppressive compounds (corticosteroid), cytotoxic drugs, cyclosporine and anti-lymphocyte antibodies (polyclonal or monoclonal), and anti-apoptosis treatment (physiologic inhibitors, viral genes and pharmacological agents), and the subject (mammals and humans) can take any of the forms previously described herein. It should also be noted that in the aforedescribed process that both the gene delivery component can be introduced into the subject and the gene can be artificially expressed as well.

[0062] In conjunction with this process, the present invention also provides a kit for producing SIDR in a subject to a gene delivery component or to an artificially expressed gene. In packaged combination or containers, the kit comprises a reagent or a combination of reagents capable of producing SIDR, and at least one other means for generating general immune suppression, or anti-apoptotic effects in the subject, or both. Buffers and instructions can also be included in the kit.

[0063] The present invention is also applicable to processes for inducing SIDR in donor (management of the donor in transplantation) against the recipient cells in order to prevent rejection of the recipient cells by the donor cells, for example, in a bone marrow transplant system. Additionally, the specific suppression of undesirable immune reactions in adults can be attained or approached using the present invention. As described earlier, this may be achieved by a variety of means either alone or in combination. In a preferred mode, SIDR can be used to suppress immune reactivity to antigens carried by recombinant viral vectors. The tolerization can be carried out before or during the course of expression of the viral vector, or it can be carried out after an immune reaction to one or more of the viral antigen has already been established.

[0064] The immunological tolerance may be induced (tolerization) by injecting viral antigens directly into the spleen or into the hepatic portal vein of the target animal (Cantor, et al., Nature 215:744-46, 1967)

[0065] The immunological determinants that are subject to the methods and compositions of immodulation of the present invention are comprised of one or more antigens. These antigens can be native or non-native with regard to the subject. They can be natural or synthetic, modified or unmodified, whole or fragments thereof. Fragments can be derived from synthesis as fragments or by digestion or other means of modification to create fragments from larger entities. Such antigen or antigens comprise but are not limited to proteins, glycoproteins, enzymes, antibodies, histocompatibility determinants, ligands, receptors, hormones, cytokines, cell membranes, cell components, viruses, viral components, viral vectors, non-viral vectors, whole cells, tissues or organs. The antigen can consist of single molecules or mixtures of diverse individual molecules. The antigen can present itself within the context of viral surface, cellular surface, membrane, matrix, or complexed or conjugated with a receptor, ligand, antibody or any other binding partner. Such antigen or antigen can be introduced to the subject alone or with agent or agents that could further contribute to delivery, uptake, stability, reactivity or targetting.

[0066] The antigen in some applications of the present invention will be introduced into the subject for

h f g e cg h e ec ec

two independent objectives. In the first instance, the antigen is introduced into the subject by means of an approriate protocol so as to produce a state of selective immune down regulation (SIDR) in said subject. In the second instance the antigen (a non native compound) is introduced into the said subject so as to provide biological function in such subject. It is further understood that there may be only immunological equivalency between the antigen or antigens used to produce SIDR and the non-native compound with biological function i.e. structurally they do not have to be identical. It is further understood that state of SIDR can be obtained in a subject wherin the subject is not only tolerant to the immunological determinant used to create the state of SIDR but it may be further tolerant to other compounds that contain the immunological determinant.

[0067] The production of SIDR in subject to noncellular immunogenic components is also an important aspect of this invention. Thus, there is provided a process for producing SIDR in any subject to a nonviable immunogenic component, which component is capable of biological function itself or is capable of interfering with a biological function in the subject. In this process, a reagent or a combination of reagents capable of producing SIDR are introduced into the subject. SIDR may be dominant as described earlier. The noncellular immunogenic component can take a number of diverse forms, including but not limited to an antibody, an antibody/antigen complex, an antibody/antigen cell matrix, an enzyme, an antitumor protein or protein inhibitor, a receptor, a hormone, a ligand, an effector and an inducer, or combinations of any of the foregoing. In the case of the antibody or antibody/antigen complex, these can be polyclonal or monoclonal in nature. Furthermore, the antibody can be to one or more epitopes on an immune cell. Merely by way of example, such epitopes can include CD2, CD4, CD8, CTLA4lg, OTK, anti-Th, or combinations thereof. See Thomson's Molecular Biology of Immunosuppression, John Wiley & Sons, Inc. (1992), for discussion of molecules and epitopes involved in immune responses. See also Tilney's Transplantation Biology: Cellular and Molecular Aspects (1996) and Kuby's Immunology, Freeman, San Francisco, (1996), Chapter 24, pp. 571-573. Both textbooks are incorporated by reference. Even further, the antibody can be directed to a number of proteins or factors including, for example, an apoptotic factor, a lymphokine, a cytokinin, and a histocompatibility factor (MHC Class I and/or MHC Class II), or any such combination. Where an enzyme is contemplated, the non cellular immunogenic component can comprise a metabolic enzyme involved in the conversion, consumption or degradation of a metabolic product or intermediate. Such metabolic enzymes are well described and representative members include L-asparaginase, superoxide dismutase, bilirubin oxidase, and adenosine deaminase or combinations thereof. See, for example, Maeda, et al., Bioconjugate Chemistry, 3:128-139 (1992) for a description of the foregoing enzymes as well as other metabolic enzymes and conjugates. That article is incorporated by reference.

[0068] Also contemplated by this invention is a kit for carrying out the SIDR process involving noncellular immunogenic components. The kit comprises in packaged combination or containers a reagent or combinations of reagents capable of producing SIDR relative to the noncellular immunogenic component capable of illiciting a biological function.

[0069] These SIDR procedures are extremely advantageous over previous procedures for inducing immune suppression in that the instant processes are specific for certain antigens. Systemic administration of small molecule immune suppressors (such as cyclophosphamide) and soluble factors such as transforming growth factor beta (TGF.beta.) or interleukin 12 (IL12) or interleukin 4 (IL4) and others (Lederr, et al., Samters Immunologic Diseases, 5th ed. Little Brown, (1995), p:129-143) will suppress certain aspects of the immune response, however, they lack antigen-specificity that the strategies listed above have. (Takahashi (1992), Ilan (1996), supra).

[0070] Another useful application of the present invention has utility relates to gene therapy. In general the use of adenovirus as a transducing virus is limited because the presence of the transducing adenovirus in the target organism leads to a cellular and humoral immune response. Host immune

h f g e cg h e ec ec

response, directed at vector antigenic determinants or a transgene product, can be a potential problem limiting the use of any viral or non-viral vectors for transferring genes into living organisms. These transgenes can include but are not limited to antibiotic-resistance genes, any selectable markers or genes that express immunologically active products. Viruses include but are not limited to HSV, HIV-based systems, retrovirus-based transducing viruses, MMLV-based systems, SV40, polyoma, HBV, EBV, VSV, Sindbis and Semliki Forest Virus, picornaviruses and other viruses that are used for transduction in animals.

[0071] The present invention also extends to non-viral gene delivery systems since these systems may also raise an immune response. These non-viral delivery systems can include but are not limited to liposomes, the various cationic and anionic lipid delivery systems, systems that induce receptor-mediated endocytosis and systems that promote the uptake of cells of nucleic acids based on the DNA or RNA transport system. In the present invention, the administration into the patient of complexes containing the antigenic carrier (consisting of fusogenic peptide, cationic lipids, anionic lipids, histones, albumin, polylysine, polysaccharides or other components) as a tolerizing agent by SIDR and/or GIS protocols or other suitable methods, will find utility in long-term gene therapy by making repeated administrations possible. In general the present invention can be used to prepare the human or animal recipient for any gene delivery that may induce an immune response.

[0072] If a patient or an animal has preexisting antibodies and cytotoxic lymphocytes directed against a specific antigen that is desired to be delivered to them, the present invention can be used to lower the titer of the antibody response, the T-cell mediated immune response or any effect of the immune response (including apoptosis, anti-idiotypic response or whatever) before the antigen is added as an adjuvant. This procedure will increase the dwell time of the reagent when it is presented subsequent to the initial presentation and also improve the ability of the complex to reach the target cell.

[0073] For example, many people have experienced an adenovirus infection sometime during their lives, and may possess a high titer of anti-adenovirus antibody in circulation. Injection of recombinant adenovirus vectors into these people may not transfer effective amounts of genetic material into these subjects because the virus will be neutralized before it reaches the target cells. Furthermore, those viruses that do reach the cells will induce an immune cell response that will lead to the elimination of the transduced target cell by normal immune surveillance mechanisms or clearance of the viral vector by the effect of the cytotoxic lymphocytes. In one of the examples that follow, it is shown that when an animal is first immunized against adenovirus such that a high titer of antibodies is induced, a subsequent SIDR protocol reduces the antibody titer to the point that recombinant adenovirus injected intranvenously can be expressed in the hepatocytes of the tolerized animal. The oral tolerizing protocol reduces the preexisting antibody levels and eliminates the synthesis of new antibodies. In time, the circulating levels of the anti-adenoviral antibodies are eliminated.

[0074] SIDR has the effect of lengthening the time of transient expression of transducing and transfection nucleic acid delivery systems. It has previously been shown that if one injects transducing adenovirus into SCID's mice, one observes transducing gene expression for 4 or 5 months (Dai, et al., Proc Natl Acad Sci USA 1995:92:1401-1405, incorporated by reference). This is a measure of the length of expression one expects from adenovirus transducing vectors in animals where the immune responsiveness is severly limited. In contrast, after injection into an immunologically functional animal which has not had previous exposure to adenovirus with an adenovirus-based transducing virus, expression of the transducing gene lasts only about two months (Dai (1995) supra). Thus, the immune response obviously shortens the period of transient expression of transducing genes of the virus.

[0075] T immunoregulatory cells (Ts cells) are induced either by certain antigen determinants or by the presence of specific allotypic or idiotypic determinants. Once these cells are induced, they act as

h f g e cg h e ec ec

memory cells capable of being reactivated throughout the lifetime of the host organism or the adaptive organism (Ilan, et al., Hepatology, 24:304,A 1996).

[0076] It should not in any way be overlooked that SIDR can be effected or obtained by means of oral tolerization. Even more significant is the principle or observation that SIDR or DIDR can also be effected through a selective immune suppressive. Such a selective immune suppressive (SIS) can comprise any of the following immune suppressors: an antibody to a T cell, an immune suppressive drug, and a cytokine or any combination thereof. The antibody to the T-cell can be representatively selected from the following: anti-CD4, anti-CD8 and OTK, or combinations thereof. Those skilled in the art will certainly appreciate that the foregoing short list of selective immune suppressives and T-cell antibodies are exemplary and by no means exhaustive.

[0077] In yet another aspect, this invention provides a process for producing selective immune down regulation in a subject to a native antigen or group of native antigens (e.g., autoimmune antigens). In this case, the process comprises subjecting said subject to at least two separate immune modulating treatments at least one of which comprises oral tolerization. As part of this process, the native antigen or group of native antigens are derived from the subject's cell or tissue, or fragments thereof, or from the subject's cell or tissue or fragments complexed with antibodies, or from partial digests of any of the foregoing. Among representative antigens or group of antigens are those selected from collagen, islet cell, liver cell, kidney cell, heart cell, pancreatic cells, spleen cell, and nucleic acid, or combinations of the foregoing. The antigens or group of antigens can also comprise a cell or tissue (for example, bone marrow) organ or components or fragments thereof. Such things can be derived or taken from the donor's skin. The second treatment in the just described process can be selected to form SIDR, GIS or anti-apoptosis. SIDR can even be used for both or all of the separate immune modulating treatments.

[0078] In the last described process, one or more cytokines can be administered to the subject, or treatments may be administered as described earlier, for example, the separate immune modulating treatments can be given repeatedly in a single dosage or single dosage period or they can be given in separate dosage periods. In addition, the treatments can be given separately or concurrently with each other. Or they can be given with partial overlap in the dosage period. It should be noted that in implementing this process and applying it to a particular case, the subject (including mammals and humans) can be sensitive or naive to the antigen or group of antigens in question. Another process unique to this invention produces immune suppression in any subject. In this instance, the process comprises administering macromolecules or compounds to the subject, the macromolecules or compounds being immunogenic or being capable of providing immune suppression, wherein the subject was previously treated to obtain SIDR to said macromolecules or compounds. This permits repeated use of the macromolecules or compounds with substantially little or no immune response.

[0079] The present invention is particularly advantageous in the field of transplantation. For example, there is provided a process for transiently producing SIDR in a subject to a specific antigen. In this process, non-native cells are transferred from a donor to the subject, wherein the donor has dominant or DIDR. For purposes of this process, the subject can be immunosuppressed prior to or during the transferring step, or even prior to and during the transferring step.

[0080] In still yet another feature, the present invention provides a process for producing SIDR in a subject to an antigen or group of antigens. Here, non-native compounds or non-native immunological reagents capable of producing immune suppression when introduced into the subject. Prior to or during or prior to and during the introducing step, the subject is exposed to the antigen or group of antigens in question, the subject having been subjected to SIDR to the non-native compounds or non-native immunological reagents. The antigen or group of antigens can be native to the subject or they can be transplanted from a donor to the subject. The SIDR can comprise antibodies to T-cells such as those

h f g e cg h e ec ec

described hereinabove, including CD4, CD8 and OTK, or combinations thereof. Other drugs or biological effectors can be administered in conjunction with this process, including one or more cytokines.

[0081] Useful and serving as another important aspect of the invention related to transplantation are other novel processes. One such transplantation process comprises introducing into a recipient subject (i) a donor liver or cells from a donor liver, and (ii) cells, tissue or organs from the donor, wherein the transplanted donor liver or donor liver cells inhibit rejection of the donor cell, tissue or organ by the recipient. The cells from the donor liver can comprise immune cells or dendritic cells. Exemplary as donor cells, tissues or organs are members selected from kidney, heart, lung, pancreas, islet cells, skin, bone or cells or tissues derived from any of the foregoing.

[0082] A further transplantation process is also provided by this invention. In this process, SIDR is established to the antigens of the donor in a recipient. The cells, tissue or organs or components thereof from the donor are then introduced into the recipient subject. This process can be supplemented with other immune modulating treatments that can be administered to either the recipient subject, the donor, or both.

[0083] A further transplantation process of this invention comprises transplanting cells, tissue or organs from a donor to a recipient subject. In this process, the recipient subject has been subjected to at least two independent immune modulating treatments, at least one of which comprises selective immune down regulation. Such cells, tissue or organs from the donor can comprise bone marrow.

[0084] Transplantation of organs (e.g. kidney, liver, heart, lungs, intestines, pancreas, skin etc.) or isolated cells or cell clusters (e.g. liver cells, pancreatic islets, etc.) or tissues derived from allogeneic living or cadaver donors require prolonged generalized immunosuppression with drugs such as cyclosporine, tacrolimus (FK506), corticosteroids (e.g. prednisolone, prednisone, methyl prednisolone), azothiaprine, cyclophosphamide, certain cytotoxic reagents such as antilymphocytic globulins (ALG) or antilymphocyte monoclonal antibodies (OKT3), etc., usually in combinations. Transplantation of organs or cells derived from other species (xenografting) is also being contemplated. Currently, systemic immunosupression is used for these procedures as well, although with limited success. Prolonged generalized immunosupression leaves the subject susceptible to infections by a wide variety of organisms, including bacteria, mycoplasma, and fungi and by viruses. These subjects are also at a much higher risk of developing malignant tumors, such as lymphomas. A strategy to decrease or eliminate the need for prolonged exposure to general immune suppresser elements will be to use a combination of a process that induces specific immune down regulation of the immune response to the immunogenic elements in the donor cells, tissues or organs and one or more of the general immune suppressor elements. One example of this will be to administer, orally, specific histocompatibility antigens or other immunogenic components of the donor cells in appropriate doses and for suitable a suitable length of time to tolerize the recipient to the allograft or xenograft. These antigens can be obtained from cells of the donor (e.g. blood cells) or expressed in vitro by recombinant technology. A second combination will be the use of two or more separate processes leading to specific immune down regulation based on the transplantation of dendritic cells (ref: Clare-Salzler, M. J., Brooks, J., vanHerle, K. and Anderson, C. (1992) J. Clin Invest. 90: 741-748) plus the oral administration of donor material described above. Two or more immunomodulatory agents may be used. By relieving the patient from the necessity of remaining on an immunosuppressant agent on a long term basis, this approach will avoid the risk of infection and lymphoma, and the other side effects of the immunosuppressive agents (e.g. nephrotoxicity).

[0085] Graft versus host disease (GVHD) is a major complication of non-solid as well as some of the solid organ transplants. In bone marrow transplant recipients, GVH is a major cause of morbidity and

h f g e cg h e ec ec

mortality. Through the present most of the measures taken to treat this disorder involved generalized immunosuppression of the patient. Attempts to delete T cells from the donor bone marrow have been made, however, they diminished the so-called graft-versus-tumor effect (GVTE) that is important to the success of bone marrow transplantation.

[0086] I In the invention, specific immune down regulation of the donor towards the recipient will be used, namely, the tolerizing of the donor against the recipient major histocompatibility, and/or other antigens will be used to render the immune cells of the donor tolerant of the recipient cellular material. The donor subject will be fed with recipient cellular material or membranes taken from various tissues most commonly involved in GVHD, such as skin, intestine and liver. Thus, by rendering the donor immunologically downregulated against the recipient, GVH can be eliminated or markedly alleviated. The graft versus tumor effect (GVTE) is not expected to be diminished as tumor cells in the recipients present different antigens than the nontumor material.

[0087] Another strategy for minimizing or eliminating transplant rejection is to use the liver as a possible tolerizing organ. It was previously shown that the liver may have a role in the induction of tolerance towards foreign antigens that are fed, or are injected into the portal vein. Specific populations of liver cells, for example, the liver dendritic cells, could selectively induce immunomodulation and/or downregulate the immune response towards foreign antigens, including allograft and xenograft-associated antigens. A donor whole liver, or a liver lobe taken from the donor, will be transplanted alongside with another solid or non-solid organ, and will induce tolerance towards the recipients. Alternatively, cells from the donor liver will be infused into the liver of the recipient through direct injection or through injection into the portal vein or the spleen and this will induce tolerance toward the donor transplanted material. This procedure will be used alone or in combination with one or more of the general immune suppressor agents or with other specific immune down regulatory agents to insure or augment the stability of cells tissues or organs transplanted into the recipient subject.

[0088] In transplantation both the recipient and donor can have specific immune down regulation. Under this condition cells, tissues and/or organs will be derived from subjects that have been previously been rendered tolerant of the donor through specific immune down regulation and placed in the recipient subject who has also in turn rendered tolerant by specific immune down regulation. This process will eliminate or diminish rejection. This double procedure can be accompanied with various immunosuppressive procedures to enhance the recipient's capacity to support the transplanted cells, tissues or organs.

[0089] Still another useful process of the present invention is one that induces tolerance in any subject. Tolerance is induced in a first subject by transferring the cells from a second subject to a first subject wherein SIDR has already been established in the second subject by the transfer of immune cells.

[0090] When there is a requirement to establish that tolerization has been conferred upon a subject, this can be accomplished either by directly assessing tolerization by a challenge type of assay or indirectly by measuring some other parameter that is associated with the induction of tolerance. Exemplary direct methods of assessing the tolerization are the in vivo introduction of the antigen into the subject and measuring the extent of an immune reaction (as described in the teaching of the present invention where antibody levels to antigens were measured) or ex vivo by removing some of the lymphocytes and assessing their ability or potential ability to react to antigen stimulus. Assessment of the extent of the immune reaction to antigen challenge can be carried out by a variety of means well known to those versed in the art. Induction of tolerance can be measured indirectly by surrogate markers that undergo changes in a subject when tolerization has occurred. Exemplary markers are the level of TGF.beta..sub.1 and other cytokines (Hancock et al., (1995) Am J. Path. 147; 1193-1197, incorporated by reference).

h f g e cg h e ec ec

[0091] In another aspect of the present invention methods are presented that can be useful in the treatment of diseases that are caused by a pathogen wherein the immune response to such a pathogen plays a significant role in the pathology of such an infection. Immune responses to virus infections, for example, involve CTL activity that acts to clear the virus from the body by killing virus infected cells or releasing appropriate cytokines. Although this response benefits the host in most viral infections, some viruses that present no direct cytopathic effects to the host can produce severe inflammatory disease as a result of the immune response. In viruses such as hepatitis B virus the immune reaction to this virus is believed to be the major cause of hepatocellular damage. The immune response can be strong enough to produce subacute or chronic hepatitis or even acute liver failure, all of which indicate a poor prognosis.

[0092] The distinction between the direct effects of the virus and the indirect effects produced by the reaction to the virus has been demonstrated in an in vivo model where HBV genomic DNA was transferred into rats (Takahashi et al., Proc. Nat. Acad. Sci., 92:1470-1474 (1995)). Although rats are not ordinarily susceptible to HBV infection, in this case infection was established as demonstrated by the presence of viral replication and expression of virus genes in the rat hepatocytes. Also, the infected rat livers showed extensive indications of hepatocellular damage that closely resembled human symptoms of hepatitis B infections. These included elevated levels of glutamic-pyruvic transaminase (a liver enzyme released from damaged hepatocytes) and histopathology indicating hepatocyte death and infiltration by lymphocytes. In contrast to the pathology produced by such an HBV infection in rats, athymic nude rats showed no such pathology, i.e., no signs of hepatocellular damage even though viremia was present and even persisted longer than in the normal rats. (Guidotti, et al., J. Vir 69:6158-6169 (1995))

[0093] In another approach, transgenic mice have been produced that contain copies of HBV DNA as part of their genetic complement (Guidotti et al., 1995). These mice contain episomal replicative HBV DNA intermediates and express HBV gene products and release viral particles that resemble those seen in a normal infection. Yet, despite the similarity to an ongoing chronic HBV infection, the livers are functional and show no signs of any defects or damage.

[0094] These studies serve to indicate that the absence of an immune response to HBV infection decreases the damaging effects of an ongoing HBV infection.

[0095] The present invention provides compositions and methods of use for the treatment of diseases caused by a pathogen that can elicit an immune response that itself is a major contributing factor to the resulting pathology. In contrast to commonly practiced therapeutic procedures for such diseases that attempt to enhance the immunological capacity to resist infection, the present invention takes an opposite approach by the use of methods that can eliminate or suppress the immune response to the pathogen. This invention thus provides a selective suppression of the immune response by tolerization to HBV wherein this can be achieved by viral components that are involved in the induction of the antiviral immune response. These include the surface protein or the viral envelope, the core proteins, the pre S1 and pre S2 proteins, as well as other virus proteins. Such compounds can be provided as their intact natural structure or as fragments thereof wherein they can be produced by chemical synthesis or by the methods of recombinant DNA. Such compounds can be provided in purified, partially purified or crude forms and can be used in intact or partially digested states. Such compounds can be administered in contact with other agents such as adjuvants or delivery systems that could be further completed or conjugated or otherwise modified to provide for stability or for more efficient administration. Other useful entities for this purpose include dead or inactivated viruses. Such compounds or entities could be administered orally, by intraportal vein inoculation, dominant transfer of tolerance and others. Also useful in the scope of the current invention are antibodies or other reagents that temporarily repress selected segments of the immune system.

h f g e cg h e ec ec

[0096] While chronic HBV infection is a deleterious disorder usually leading to end stage liver disease, most patients cannot benefit from antiviral agents such as interferon. The present invention can permanently depress the immune system and thereby abrogate any hepatocellular damage while other therapeutic agents may or may not be used to provide treatment for the viral infection itself. Because the majority of the hepatocellular damage is a result of the immune response, and the virus itself is non cytopathic, the viremia should not be harmful. Tolerization to eliminate or significantly suppress the pathology associated with an HBV infection can therefore essentially transform a chronic HBV patient into a "healthy" carrier whose response to the infecting HBV is similar to the vast majority of the HBV infected patients who carry the virus for life without the development of any major complications. In these cases the immune system cannot clear the virus, and the patient can be considered as tolerized to HBV. Chronic HBV patients, in contrast, are patients in whom the immune system, as a consequence of attempting to clear the virus, damages virus-infected as well as non-infected hepatocytes. In this case also, transforming of a chronic HBV patient into a "healthy" HBV carrier by tolerizing against the virus can alleviate or even cure the hepatocellular damage.

[0097] Tolerization to HBV can also be useful for eliminating or reducing HBV recurrence in patients who have received liver allografts. Currently, HBV recurrence is the major obstacle for liver transplantation in patients with HBV related illnesses; the rate of recurrence in such patients is 20-90% within the first year post transplantation. Infection is considered to result from HBV infected bone marrow and peripheral blood lymphocytes that appear to be major reservoirs for the virus. HBV recurrence in post liver transplantation is usually associated with severe liver injury that is considered to be immune mediated and which normally leads to a rapid deterioration in liver functions and death.

[0098] While the exact mechanisms involved in the induction of tumors by the virus are unknown, it appears to involve integration of parts of the virus into liver cells genome as well as involvement of a defective antiviral immune response. Thus, in cases where the immune response to the HBV may have a role in the tumurogenesis of the virus, tolerization to HBV can provide useful benefit to the prevention of development of hepatocellular carcinoma.

[0099] The present invention provides compositions and methods of use for therapeutic agents that have antigenic properties wherein such agents can be used without the risk of an unwanted immune response.

[0100] Specific immune responses to foreign and infectious agents are recognized as being both necessary and beneficial to the maintenance of a healthy subject. In fact, induction of an immune response to an infectious agent (vaccination) and promotion of an effective immune response in the face of infection are desired and recommended therapeutic regimens. However, in certain instances, an immune response to an infectious agent leads to undesirable consequences such as an autoimmune response or direct or indirect destruction of infected or uninfected cells and tissues. For example, in the case of HIV infection, HBV or HCV infection or rheumatic heart disease, such undesirable immune consequences are observed. In spite of such a problem, it is not recommended to limit or inhibit the immune response directed against the infectious agent. As such, there are no effective therapeutic regimens that address both the undesirable immune consequences as well as depropagation of the infectious agent.

[0101] It is another aspect of this invention to overcome the limitions that exist in treating the condition of these infections by uncoupling the immunological response from the propagative aspect of the infection and addressing each one independently. According to the teachings of this invention, the subject (patient) is treated with one or more immunological modulation protocols that would lead to a state of SIDR and GIS or a combination thereof while, if desired or necessary, addressing the propagative aspects of an infection by the use of appropriate compounds directed against the pathogen such as anti-bacterial, anti-fungal or antiviral agents including viral protein inhibitors or viral replication

h f g e cg h e ec ec

inhibitors.

[0102] Although HIV infection is noted for the breakdown of the immune system, there is evidence that the loss of CD4.sup.+ cells is not due completely to the direct effect of viral infection but there also might be an auto-immune component that is responsible for CD4.sup.+ cell depletion. This was originally suggested in 1986, (Ziegler, J. L. and Stites, O., Clin Immunol. Immunopathol., 41;305) and support for this concept has continued to accumulate. For instance, although chimpanzees can be infected readily by HIV, there are no signs of progression into AIDS as seen in humans. An important difference that has been noted between HIV infections of humans and chimpanzees are the absence of cytotoxic T lymphocytes (CTLs) in the latter (Zarling, et al., (1990), J. Immunol. 144; 2992-2998. In contrast, infections in humans resulted in the production of CTLs that are capable of lysing uninfected CD4.sup.+ cells from humans and chimpanzees. Also a correlation between the level of autoantibodies and the level of depletion of CD4.sup.+ cells has been observed (Muller, C., Kukel, S., and Bauer, R. (1993) Immunology 79; 248-254, incorporated by reference herein).

[0103] Thus, even while therapeutic means are employed to block viral replication, there can be a system that maintains ongoing destruction of CD4.sup.+ cells. In such a case, therapeutic benefits are achieved by applying the teachings of this invention. One or more immune modulation protocols is administered to the patient to induce a SIDR or GIS state against the viral antigen or viral antigen complexed with antibody or viral antigen complexed with cell receptors or viral antigen within the cell matrix.

[0104] The patient, in such a state will exhibit reduction, inhibition or elimination of an autoimmune response. The patient is maintained on an anti-HIV regimen which includes protease inhibitors and/or inhibitors of viral replication. Such a protocol could be supplemented with the appropriate cytokines (reviewed by A. Fauci, (1996) Nature 384; 529-534, incorporated by reference herein) or antibodies directed against specific T-cells.

[0105] In patients with HIV infection, the circulating antibodies do not provide protection to the host. In addition, both the viral antigen and anti-idiotypic antibodies that mimic the antigens, can bind to CD4.sup.+ cells and will interfere with the functioning of these cells and may induce their apoptosis. The component of the HIV particle that is responsible for binding to the CD4.sup.+ receptor is the viral gp120 protein. Vaccines have been made that are based upon this protein to elicit an antibody response to the HIV (Eron et al. (1996) The Lancet, 348; 1547-1551). No effects were seen, however, in the progression of the disease.

[0106] Based on the teachings of this invention, oral administration of HIV proteins and/or components and its complexes and conjugations to the corresponding antibodies or receptors (CD4) or cell membranes containing such an HIV antigen,- in appropriate doses and duration will reduce the antibody levels. The patient in this stage will demonstrate reduction or inhibition of the autoimmune response. Furthermore, these patients will show an improved immune competence. The HIV load will be reduced by cotreatment with currently available drugs or by newer methodologies, including genetic antisense therapy. This procedure can be generalized to any virus that produces interfering antibodies during the humoral response to the viral antigens. Viruses that may fall in this category include those viruses that evoke an immune response in response to infection and yet the humoral or cell-mediated immune response seems not to be effective against the spread of the virus. Candidate viruses for this type of therapy include HIV-1, HIV-2 and HTLV-1.

[0107] In the present example, viral protein (gp120 or its fragment) is administered to an infected patient as a means of oral tolerization to the HIV antigen. Details of methods of production of a viral protein and administration of proteins in an oral tolerization program are described in the teachings of this

h f g e cg h e ec ec

invention and in "Oral Tolerance: Mechanisms and Applications" H. L. Weiner and L. F. Mayer, eds. (1996) The New York Acadamy of Sciences New York, N.Y., the contents of which are fully incorporated herein by reference. When the patient still has a functional immune system at this stage of the disease, there should be tolerization to the viral antigen that should diminish the rise in an autoimmune response that could be generated by this protein.

[0108] This dual treatment protocol would allow blocking of viral replication and propagation while keeping the autoimmune system dormant. If the patient is in a GIS state during the course of treatment, then, after effective anti-viral propagation therapy, the immune suppression can be released allowing restoration of the immune system.

[0109] An aspect of this invention is to use immunological modulation protocols taught by this invention to block or to diminish the number of target cells available to the HIV so that the opportunity of the virus to propagate is diminished, while the patient is maintained on an anti-viral protocol. If a method is used to reversibly block the immune system by reducing the number of T4 cells, or other HIV target cells or their rate of proliferation, by the use of immunomodulation protocols and reagents, or by blocking of the CD4.sup.+ or other target cell receptors (treatment with anti-CD4.sup.+ and/or OKT.sub.3 and/or anti-macrophage antibodies e.g., CD14.sup.+), the number of available cells for infection by the HIV should be decreased or diminished, allowing a method of increasing the impact of anti-viral therapeutic agents.

[0110] Whereas efforts to treat a variety of diseases by the administration of compounds such as proteins and polypeptides can provide effective therapy, useful benefit can be diminished by immune responses to the therapeutic agent, especially when the therapeutic agent is non-native to the patient. The present invention overcomes these limitations and opens pathways to the use of a broad range of such agents. This advantage is achieved by the use of immune modulation as a means of providing for effective in vivo treatment with such compounds. Compositions and methods of use are provided herein for transient or prolonged use of such therapeutic agents introduced into the body without the risk of an unwanted adverse immune response.

[0111] Therapeutic agents that can be utilized with the compositions of the present invention are non-native, non-viable, have the potential of being immunologically recognized and can perform a biological function wherein they can be synthetic, natural, cloned, modified or an analogue that in the body, indirectly or directly, whether intracellular or extracellular, can perform a biological function or interfere, inhibit or enhance a biological process. Such agents, referred to herein as non-native active compounds, include: enzymes, antibodies, ligands, co-factors, hormones, cytokines, lymphokines and factors that induce or inhibit apoptosis and others.

[0112] Such non-native active compounds can, in the body, perform a biological function or can interfere with, inhibit or enhance one or more biological functions including such biological functions that are artificially provided (such as by the methods of gene therapy). Non-native active compounds include enzymes such as non-native factor IX that can provide for this missing element in certain types of hemophilia, bacterial bilirubin oxidase that can act to reduce bilirubin concentrations, non-native superoxide dismutase that can remove free radicals in tissues such as cardiac tissue that has been traumatized as a result of myocardial infarction, E. coli aspagaginase that modifies L-aspagagine in tumors thereby inhibiting tumor growth, bovine adenosine deaminase for treatment of ADA deficiency and polymerases, integrases and other enzymes that can serve as components of a gene delivery construct.

[0113] Antibodies can be useful as non-native active compounds wherein they can be monoclonal, polyclonal or wherein they can be intact natural proteins or fragments thereof and can be modified such

as in a chimera with one or more other antibodies or proteins. Antibodies useful as non-native active compounds include monoclonal antibodies such as OKT3 that can ablate peripheral lymphocytes for the purpose of combating acute allograft rejection, and antibodies to CD8 cells as a means of controlling the killing of CD4+ cells in HIV-1-infected individuals (U.S. Pat. No. 5,424,066) as well as other antibodies such as anti-CD4, anti-CD8, anti-Thy and anti-NK that can be used to promote skin graft tolerance (Zhao, Y. et al. 1996 Nature Med 11: 1211-1216). Both the foregoing patent and the article are incorporated herein by reference. Other useful antibodies include those that block histocompatibility determinants in donor organs, antibodies that recognize and bind to certain tissues and organs and can thus be used for radioimaging, and antibodies that bind to certain tissues and organs and that can be modified with certain cytotoxic agents, such as risin, to provide selective cell killing.

[0114] Non-native hormones such as estrogens and androgens can provide such useful functions as inhibiting apoptosis. Non-native cytokines or lymphokines can provide useful benefit.

[0115] Such non-native active compounds can be provided in their natural structure or as fragments thereof wherein they can be natural or can be produced by chemical synthesis or by the methods of recombinant DNA. Non-native active compounds can be provided in purified, partially purified or crude forms and can be used in intact or partially digested states. Non-native active compounds can be administered in contact or in concert with other agents such as adjuvants or delivery systems that could be further complexed or conjugated with a recipient antibody and/or a receptor, or with a cell matrix. Such compounds could be modified to provide a longer half-life in the body (Maeda, H. et al. 1992 Bioconj Chem 3: 128-139). Non-native active compounds can be modified with ligands, such as biotin, in order to provide binding cells (See Example 7 below)

[0116] The present invention provides for the administration of non-native active compounds without the risk of an immune response that could diminish the effectiveness of such treatment whether such treatment is transient or whether such treatment is made repeatedly over a prolonged period. The present invention thus provides for the effective biological function of these non-native active compounds without interference by the body's immune response. This can be achieved by the use of immune modulation as provided in this invention wherein it can be used as general immune suppression for transient or short term treatment and/or by tolerization, provided by selective immune down regulation, for prolonged treatment. In some cases a combination of two or more such immunomodulation regimens can be advantageous. Such treatments can be applied prior to and/or during the course of administration of non-native active compounds. Thus, for example, in order to prevent the development of unwanted immune responses to non-native active compounds in the early stages of long-term administration, it may be desirable to commence SIDR measures prior to treatment. Alternatively, in the absence of any prior measures to establish immunomodulation, SIDR measures can be performed simultaneously with administration of a non-native active compound. In this case, in order to prevent the development of immune responses during the early stages of treatment, general immune suppression could be used during this period. In cases where an individual has a preexisting capability to mount an immune response to a non-native active compound SIDR can commence prior to administration of such a compound. Alternatively, in the absence of any prior measures to establish immunomodulation, SIDR can commence simultaneously with administration of a non-native active compound. In cases where one or a very few treatments are administered over a short period of time general immune suppression may be useful without the requirement for tolerization.

[0117] For instance, recombinant adenoviruses are being used by many investigators for somatic gene therapy (Ali, et al., Hepatology, 24:304,A 1996, Gene Therapy 1:367-384; Jaffe; et al., 1992, Nat. Genet. 1:372-378). However, the expression of foreign genes delivered by these vectors is of limited duration both because of the episomal nature of adenoviruses (Prevec, et al., J Gen Virol 1989:70: 429-434; Horwitz, et al., Virology. Raven Press: New York, 1990:1679-1721), and, more importantly,

h f g e cg h e ec ec

because of the host humoral and cellular immune response (Yang, et al., J Virol 1995:67:2004-2015). All of the foregoing articles and book are incorporated by reference. Host cytotoxic lymphocytes (CTL) against adenovirus infected cells may clear the adenovirus infected cells, reducing the duration of transgene expression in viva after the initial injection of the virus. Neutralizing antibodies that appear in response to the initial exposure to adenoviral proteins prevent effective gene transfer to hepatocytes upon reinjection of the virus.

[0118] Recombinant adenoviruses are generated by insertion of the target gene into the E1 region of the viral genome, thus disrupting the E1 gene and rendering the virus replication defective (Graham, et al., Methods in Molecular Biology. The Humana Press: Clifton, N.J., 1991:109-128.). Attempts have been made to further cripple the adenoviral vector by using a virus containing a mutation in the E2a region that results in the expression of temperature-sensitive DNA binding proteins. However, these "second generation" adenoviruses are still able to invoke a potent anti-viral immune response (Yang, et al., Nature Genetics 1994:7: 362-369; Engelhardt, et al., Proc Natl Acad Sci, USA 1994; 91:6196-6200.). It is likely, therefore, that the antigenic load in the input recombinant virus is sufficient to produce this immune response. Thus other mechanisms of modulating the anti-viral immune response need to be sought.

[0119] Studies in nude and SCID mice with defects in T or in both T and B cell functions, as well as the use of several immunosupressed regimens including FK506, cyclosporine and cyclophosphamide have shown that a longer duration of gene expression can be achieved with systemic immunosuppression (Ilan, et al., J Hepatol (abstract) 25:73A; Dai (1995) supra; Fang, et al., Human Gene Therapy 1995:6:1039-1044). However, the applicability of these methods in humans is limited because of the general immunosuppressive state they induce in the recipient. Moreover, these methods would not allow gene transfer in the presence of preexisting neutralizing antibodies against the virus.

[0120] The examples that follow are given to illustrate various aspects of the present invention. Their inclusion by no means is intended to limit in any way the scope of this invention as more particularly set forth in the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Examples

Example 1

Establishment of Oral Tolerance to Recombinant Adenovirus Antigens

[0121] Enteral exposure to foreign antigens has been shown to induce antigen specific tolerance by clonal inactivation of antigen specific T cells or by the induction of regulatory cells secreting factors that suppress the generation of antigen-specific effector cells (Weiner, et al., 1994, Proc. Natl. Acad. Sci. USA 91:10762-10765; Vandenback, et al., J. Immunol. 153:852-9 (1994); and Hirahara, et al., J. Immunol. 154:6238-6245 (1995)). Therefore, in this example, high dose and low dose tolerizing regimens were used and it was demonstrated that induction of oral tolerance to adenoviral antigens can be used to abrogate the host anti-adenoviral immune response in a model system that employs Gunn rats, a strain that lacks hepatic bilirubin uridine-diphosphoglucuronate glucuronosyltransferase-1 (BUGT.sub.1).

[0122] Animals:

[0123] Inbred Gunn and congenic normal Wistar RHA rats were bred and maintained in the Special

h f g e cg h e ec ec

Animal Core of the Marion Bessin Liver Center of the Albert Einstein College of Medicine. The rats were maintained on standard laboratory chow and kept in 12 hr light/dark cycles.

[0124] Plasmids:

[0125] pJM17 was kindly provided by Dr. F. L. Graham, McMaster University, Hamilton, Canada.

[0126] Generation of Recombinant Adenovirus:

[0127] Two recombinant adenoviruses, Ad-hBUGT.sub.1 and Ad-LacZ expressing human bilirubin-UGT.sub.1 and E. Coli b-galactosidase, respectively, were generated as described previously (Takahashi (1996) supra). In brief, transcription units consisting of the promoter and enhancer sequence for the immediate early gene of cytomegalovirus (CMV), the structural region of human BUGT.sub.1 or E. Coli b-galactosidase, and the polyadenylation signal from bovine growth hormone, were recombined into the E1 region of human Ad-5 to produce replication-defective "first generation" adenoviruses. For large-scale preparation, the recombinant adenoviruses were grown on 293 suspension cells and purified from cell lysates by two consecutive CsCl density gradient centrifugations, and stored in 30% glycerol at -20.degree. C. Virus was dialyzed overnight at 4.degree. C. against an isotonic solution containing 135 mM NaCl, 5 mM KCl, 1 mM MgCl.sub.2, 10 mM Tris-HCl, pH 7.4, and 10% glycerol, and sterilized by filtration through 0.45 um filters before use (Horowitz (1990) supra).

[0128] Preparation and Administration of Viral Protein Extract:

[0129] The CsCl gradient supernatant, containing major adenoviral structural proteins, mainly fiber, hexon and penton, was collected and the protein concentration was determined (Horwitz, et al., Virology 39: 682-694 (1969); Mazel, et al., Virology 36:126-136 (1968)). Under ether anesthesia, a polyethelene cathether (PE10) was inserted into the stomach through a midline incision. The tube was advanced into the duodenum and affixed to the stomach wall. The other end of the catheter was exteriorized at the dorsal aspect of the neck by subcutaneous tunneling. Each of the rats was kept in a separate cage throughout the study. The protein extracts were introduced through the catheter every other day for 21 days (a total of 11 doses).

1TABLE 1 Experimental groups: Recombinant Recombinant virus virus injected on first injected on second Group Antigen fed and dose: injection injection A adenoviral proteins: Ad-hBUGT.sub.1 Ad-hBUGT 1 mg per rat per day B adenoviral proteins: 1 Ad-hBUGT.sub.1 Ad-LacZ mg per rat per day C bovine serum albumin Ad-hBUGT.sub.1 (C1) Ad-hBUGT.sub.1 1 mg per rat per day (5 rats) (C2) Ad-LacZ (5 rats) D None Ad-hBUGT.sub.1 Ad-hBUGT.sub.1 E adenoviral proteins: Ad-hBUGT.sub.1 Ad-hBUGT.sub.1 50 mg per rat per day (n = 5), or 100 mg per rat per day (n = 5)

[0130] Ad-hBUGT.sub.1 and Ad-LacZ Injection into Gunn Rats:

[0131] Five groups of Gunn rats, consisting of 10 animals in each group, were studied. Groups A and B included rats that were fed with adenovirus protein extract at a dose of 1 mg/rat every other day followed by two injections of Ad-hBUGT.sub.1 (5.times.10.sup.9 pfu) on days 1 and 98 (Group A), or Ad-hBUGT.sub.1 on day 1 followed by Ad-LacZ on day 98 (Group B). Two groups of rats were used as controls: Group C received bovine serum albumin 1 mg/day and then received viral injections as described for Group A (5 rats, group C1) or for Group B (5 rats, group C2). Group D did not receive any oral proteins and was injected with Ad-hBUGT.sub.1 similarly to Group A (Table 1). In order to evaluate the mechanism of the tolerance, and distinguished between induction of suppressor cells and clonal inactivation Group E rats were fed with 50 mg/day (5 rats), or 100 mg/day (5 rats) of the viral protein extract (high dose regimen), followed by two injections of Ad-hBUGT.sub.1.

h f g e cg h e ec ec

[0132] Evaluation of Immune Tolerance

[0133] Liver Histology:

[0134] For evaluation of the degree of hepatic inflammation, liver biopsies were performed one week after the second injection in 2 rats from each of the treated groups and kept in 10% formaldehyde. Paraffin sections were then stained with hematoxylin-eosin according to standard procedures. The sections were graded for hepatic inflammation as follows: Grade 0: normal; Grade 1: mild periportal or focal lobular lymphocytic infiltration; Grade 2: extension of lymphocytic infiltration into the lobules and "piece-meal necrosis"; and Grade 3: disruption of the lobular architecture by "bridging necrosis" and extension of lymphocytic infiltrates from portal to central, portal to portal and central to central zones.

[0135] Serum Alanine Amino Transferase (ALT) Levels:

[0136] As a measure of the degree of hepatic inflammation, ALT levels were quantified using a commercially available kit (Sigma, St Louis, Mo.).

[0137] Neutralizing Anti-Adenoviral Antibodies:

[0138] Anti-adenoviral neutralizing antibodies present in the sera of treated rats were measured on days 28, 78, 112, and 196 in all rats that received Ad-hBUGT.sub.1 injection. 293 Cells were seeded at a concentration of 3.times.10.sup.4/well in 96 well plates, and cultured until 90% confluency. Ad-LacZ was diluted in cell culture medium to give 3.times.10.sup.5 pfu/10 ml. Serum samples were heat inactivated at 55.degree. C. for 30 min and diluted in medium in twofold steps. 100 ml of each serum dilution was mixed with 5.times.10.sup.5 pfu of the recombinant virus, incubated at 37.degree. C. for 90 minutes, and applied to the nearly confluent 293 cells for 10-14 hours. The supernatant containing serum and virus was then replaced by RPMI medium with 10% FCS for 18 hours. Cells were fixed and stained for b-galactosidase expression. In the absence of neutralizing antibodies all of the cells stained blue. The neutralizing antibody titer for each serum sample was reported as the highest dilution at which less than 25% of the cells stained blue.

[0139] Cytotoxic T Lymphocyte Assay:

[0140] Two rats from each group were studied on days 28, 78, 112 and 196. Spleens were removed under anesthesia from each of two rats at each time point, and the animals resutured. The organs were gently disrupted using a rubber policeman. Red blood cells were removed using lysis buffer containing 0.17M NH.sub.4Cl at pH 7.4 (1 ml/spleen) for 2 minutes. Lymphocytes were spun down and plated at 5.times.10.sup.7 cells per 5 ml in RPMI medium with 10% FCS. Cells were then restimulated with the recombinant adenovirus Ad-hBUGT.sub.1 (1-10 pfu/cell) for 4-5 days. Adenovirus infected primary hepatocytes, harvested by collagenase perfusion of the liver (Seglen, Methods in Cell Biology, 1976)), were used as target cells for the effector lymphocytes and were plated on collagen coated 6 well plates in Chee's medium (2.times.10.sup.8 cells/well). Stimulated effector cells were harvested, counted and added to the primary hepatocyte cultures at a ratio of 50-100:1 and incubated at 37.degree. C. for 5 hours. Hepatic cell lysis was measured by collecting the medium and measuring alanine aminotransferase (ALT) levels using a commercially available kit (Sigma, St Louis Md.) with the following modifications: the ratio between reagent and test medium was changed from 10:1 to 1:1, and the reaction time before the first spectrophotometric reading was 90 seconds, followed by a reading every 30 seconds up to 5 minutes. ALT levels were then calculated according to the manufacturers' formula and expressed in international units. Background ALT levels were determined by measurements of the ALT levels in the supernatants of dishes containing adenovirally infected hepatocytes and

h f g e cg h e ec ec

lymphocytes from naive rats. CTL activity was expressed in IU of ALT averaged from 6 wells after subtraction of background levels.

[0141] Results

[0142] Evaluation of Immune Tolerance

[0143] Liver Histology:

[0144] Liver biopsies from two rats in each group examined 24-72 h after the second injection showed minimal or no periportal or lobular lymphocytic infiltration in recipients that were tolerized by enteral administration of adenoviral proteins (group A). In contrast, a severe inflammatory reaction (grade 3) was observed in liver specimens taken from rats that were given BSA or no protein prior to the injection of the virus (groups C, D) and rats that received high doses of adenoviral antigens (group E) (not shown).

[0145] Serum ALT Levels:

[0146] In the group A rats that were tolerized with adenoviral proteins, serum ALT levels increased only minimally after each of the three injections (96-110 IU; normal levels before any manipulation were 60-75 IU). In groups that received BSA or no protein (C and D), ALT levels increased to 168 IU after the first injection, and to 212 IU after the second injection.

[0147] Neutralizing Antibodies:

[0148] After injection of Ad-hBUGT.sub.1 in rats that had received BSA or no proteins (Groups C, and D, respectively) high titer (>1:2816) antibodies appeared during the first month. In contrast, in the tolerized rats (Group A), neutralizing antibodies were undetectable in 80% of the recipients. The-remainder exhibited low titers of the antibody (<1:16) (FIG. 5). Rats that developed the low titer antibodies had similar hypobilirubinemic responses to the second injections of Ad-hBUGT.sub.1 as did the rats that had no detectable antibodies.

[0149] Cytotoxic T Lymphocyte Response:

[0150] Cytotoxic T cells were tested against adenovirus infected rat hepatocytes four times throughout the study. Measurement of the amount of ALT released from the hepatocyte targets into the media was used to assess the CTL response. ALT levels in the media were below 80 IU in all tolerized recipients (groups A and B), but exceeded 450 IU in non-tolerized rats (groups B and C) (FIG. 6).

[0151] Effect of Antigen Dose:

[0152] To evaluate the relationship between the dose of adenoviral proteins and induction of tolerance, 10 rats in group E were fed with the viral proteins at higher doses (50-100 mg/day). In these rats serum bilirubin levels and HPLC analysis of pigments excreted in bile indicated that the second recombinant adenoviral injection failed to achieve gene expression or a metabolic effect. The anti-adenovirus immune response in this group was similar to that in rats that were administered BSA or no protein at all (groups C and D). Thus although no evidence for tolerance was observed using higher doses of the antigen, the administration of low dose feeding of adenoviral proteins markedly inhibited, both humoral and cellular host immune response to the recombinant adenovirus containing the human BUGT.sub.1 gene. High dose feeding, which has been shown to induce anergy or deletion of antigen-reactive T cells, was found to be ineffective. In contrast, oral tolerization with low dose feeding of adenoviral protein

h f g e cg h e ec ec

extracts, markedly inhibited both the humoral and cellular host immune response to the recombinant adenovirus containing the human BUGT.sub.1 gene.

Example 2

Oral Tolerization to Recombinant Adenovirus Prolongs Expression Time and Permits Readministration

[0153] The animals used in Example 1 were also used to evaluate the effect of an oral tolerization regime on the length of expression from recombinant adenoviruses

[0154] Assessment of Transgene Expression.

[0155] b-Galactosidase Expression:

[0156] Gunn rats from groups that received Ad-hBUGT.sub.1 as the first injection and Ad-LacZ as the second injection with (group B) or without (group C2) previous administration of adenoviral proteins underwent liver biopsies. Specimens were frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C.), in a dry ice cooled methyl butane bath. Frozen Cryostat sections (10 um) were fixed for 5 minutes at room temperature in freshly prepared 1% glutaraldehyde in PBS. .beta.-Galactosidase activity was detected by immersing the section into 5-bromo-4-chloro-3-indol-b-galactopyranoside (X-Gal) staining solution (5 mM K.sub.4FeCN, 5 mM K.sub.3FeCN, 1 mM MgCl.sub.2, containing 1 mg of X-Gal per ml) for 8-15 hours at 37.degree. C. Sections were briefly counterstained with eosin, then dehydrated and mounted.

[0157] DNA Analysis Using PCR:

[0158] To detect the presence of the human BUGT.sub.1 gene in the host liver, DNA was extracted from RNase treated tissue homogenates. Two rats from each of the experimental groups A and E, and the control groups C1 and D, were tested 3 days after the second Ad-hBUGT.sub.1 injection. DNA was subjected to amplification by polymerase chain reaction (PCR) using primers (sense: 5'AAGGAAAGGGTCCGTCAGCA 3' from nt 141 to nt 160, antisense: 5' CCAGCAGCTGCAGCAGAGG 3' from nt 441 to nt 462) designed to amplify a 321-bp segment of the the unique exon 1 (exon 1*1) of the human BUGT.sub.1 gene. PCR amplification was performed using the following protocol: 94.degree. C. for 30 sec. 58.degree. C. for 30 sec, and 72.degree. C. for 1 min.times.30 cycles.

[0159] Expression of Human-BUGT.sub.1 Protein:

[0160] For determination of the expression of hBUGT.sub.1, liver specimens were taken from two rats in experimental Group A and control Group C1 five days after the second viral injection. Tissue homogenates (200 mg/ml) were prepared in 0.25 M sucrose/10 mM Tris-HCl, pH 7.4 using a glass homogenizer fitted with a motor-driven teflon pestle. For immunoblot analysis, proteins (100 mg/lane) were resolved by electrophoresis on SDS-polyacrylamide (7.5%) gels and electroblotted to nitrocellulose membranes. The membranes were probed with a monoclonal antibody WP1 directed at the common carboxyterminal domains of UGT isoforms expressed by hBUGT.sub.1, followed by peroxidase conjugated goat anti-mouse IgG F'ab fragment second antibody (Sigma, St. Louis, Mo.) and substrate (Peters, et al., Gastroenterology 93:162-169 (1987); Towbin, et al., Proc. Natl. Acad. Sci. USA 76:4350-4354 (1979)). Equal protein loading in all lanes was assured by performing the electroporesis on an identical SDS-polyacrylamide gel and staining the protein bands with Coomassie brilliant blue.

[0161] Assay for BUGT.sub.1 Activity Towards Bilirubin:

h f g e cg h e ec ec

[0162] The enzyme assay was performed on homogenates of liver specimens from two rats from each experimental group that received Ad-hBUGT.sub.1 injection (A, E, C1 and D), 20 days after the first and second injection, and from all other rats at the termination of the experi ments. The assay method was as previously described, using 80 mM bilirubin as the aglycone (Trotman, et al., Anal Biochem 121:175-180 (1982); Roy Chowdhury, et al., Hepatology 1:622-627 (1981)).

[0163] Determination of Serum Bilirubin Levels:

[0164] Serum bilirubin levels were measured according to Jendrasik and Grof in all groups every 10-14 days throughout the study period (Trotman (1992) supra).

[0165] Bile Pigment Analysis:

[0166] For definitive demonstration of bilirubin glucuronidation in selected rats, bile was collected through a polyethylene bile duct cannula and bilirubin glucuronide excreted in bile was analyzed by HPLC using a uBondapak C-18 column (Millipore-Waters, Milford, Mass.) as described previously (Roy Chowdhury (1982) supra). Bile was analysed in two rats from experimental groups that received adenoviral proteins at 1 mg/day (group A), 50 mg/day or 100 mg/day (group E) BSA 1 mg/day (groups C1) or no protein at all (D), 20 days after the first and second injections of the recombinant adenovirus. All other rats had bile pigment analysis at the termination of the experiments.

[0167] Results

[0168] Expression of b-Galactosidase Activity:

[0169] For histochemical staining, liver biopsies were performed, 7 days after Ad-LacZ injection from liver specimens of Gunn rats that received Ad-hBUGT.sub.1 as the first injection and Ad-LacZ as the second injection with (group B) or without (group C2) prior administration of adenoviral proteins. Biopsies were performed on two rats in each group. Histochemical staining of cryostat sections (10 mm) showed that the great majority of hepatocytes stained positive for b-galactosidase activity after the injection in rats that had been administered the adenoviral proteins (group B), while only 5% of hepatocytes stained positive, in livers from rats that were given BSA (group C2) (FIG. 1).

[0170] Expression of h-BUGT Gene After Recombinant Adenoviral Injection into Gunn Rats.

[0171] DNA Analysis Using PCR:

[0172] Presence of hBUGT.sub.1 DNA in the liver of Gunn rats that received Ad-hBUGT.sub.1 with (group A) or without (groups C1 and D) without prior enteral administration of adenoviral proteins was evaluated by PCR after the second AdhBUGT.sub.1 injection. A DNA fragment of 321 bp was seen only in rats from group A, while both control groups C and D, were negative. Normal human liver and liver from an untreated Gunn rat were used as positive and negative controls, respectively (FIG. 2)

[0173] Expression of Human-BUGT.sub.1 Protein:

[0174] Liver specimens were collected from two rats in groups A and C1, 5 days after the second Ad-hBUGT.sub.1 injection. Immunoreactive 52 kDa bands, corresponding to hBUGT.sub.1 were observed in Gunn rats that were given two injections of Ad-hBUGT.sub.1 after the administration of adenoviral antigens (group A) but not in the group that received the virus injections after enteral administration of BSA (group C) (FIG. 3). Normal human liver and untreated Gun rat livers were used as positive and

h f g e cg h e ec ec

negative controls respectively.

[0175] BUGT.sub.1 Activity in Vitro:

[0176] UGT activity toward bilirubin was undetectable in untreated Gunn rats. In homogenates of normal human specimens obtained from cadaver donor organs, the BUGT.sub.1 activity was 78.+-.26 nmol/mg liver weight/min; (mean.+-.SEM, n=6). In liver homogenates from two rats that received Ad-hBUGT.sub.1 injections after enteral administration of adenoviral proteins (group A), bilirubin-UGT activity was 80 and 85 nmol/mg liver wet weight/min, 20 days after the first Ad-hBUGT.sub.1 injection, and was 88.+-.20 nmol/mg liver wet weight/min (mean.+-.SEM, n=5) 20 days after the second injection. In the BSA-fed rats, (groups C1 and D), bilirubin-UGT activity was undetectable after the second injection of ad-hBUGT.sub.1.

[0177] Serum Bilirubin Levels:

[0178] Bilirubin levels were measured every 10-14 days. A marked decrease in bilirubin levels occurred after each Ad-hBUGT.sub.1 injection in Gunn rats that were tolerized by the administration of adenoviral proteins (group A), with levels reaching as low as 1.83, and 1.78 mg/dl after the first and second injections, respectively (FIG. 4). Bilirubin levels remained low for over three months after each injection, and then increased gradually. In contrast, in BSA-fed Gunn rats (Groups C and D) the first Ad-hBUGT.sub.1 injection reduced serum bilirubin levels to 2.73 mg/dl for only 4 weeks, followed by a progressive increase to preinjection levels. Subsequent Ad-hBUGT.sub.1 injections had no effect on serum bilirubin concentrations in these groups.

[0179] Bile Pigment Analysis:

[0180] HPLC analysis of bile collected from two rats from Ad-hBUGT.sub.1-treated rats (groups A, C1, D and E) 20 days after the first injection of Ad-hBUGT.sub.1 showed excretion of bilirubin mono- and diglucuronide. The two glucuronides accounted for more than 95% of the bile pigments, less than 5% being unconjugated bilirubin. This profile was similar to that seen in normal Wistar rats. A similar pattern was seen in rats tolerized by enteral administration of 1 mg/day adenoviral proteins (group A) 20 days after the second Ad-hBUGT.sub.1 injection. In rats that received BSA (group C), no protein (group D) or high doses of adenoviral proteins (50-100 mg/day, group E), bile pigment analysis after the second Ad-hBUGT.sub.1 injection did not show significant amounts of conjugated bilirubin in the bile. Gunn rats injected with Ad-LacZ did not excrete bilirubin glucuronides in bile. Chromatographic profiles in bile from these rats resembled that from untreated Gunn rats.

[0181] Discussion

[0182] Oral tolerization prolonged the transgene expression, as shown by the longer duration of the hypobilirubinemic effect. However, the effect was not permanent, as indicated by the gradual increase of serum bilirubin levels between day 14 and 98 after the first Ad-hBUGT.sub.1 injection. A similar decay of transgene effect was seen after induction central tolerance to recombinant adenoviruses induced by injection of the virus during the newborn period (Takahashi (1996) supra). The decline of transgene effect seems to have resulted from the degradation of the episomal adenoviral DNA, rather than the loss of tolerance, because there was no antiviral CTL activity in the host during this period.

[0183] In conclusion, this example shows the potency of low dose oral viral antigen administration in down-regulating the antiviral immune response.. In addition, this example demonstrates that after the activity of the desired gene product is diminished, the recombinant adenovirus can be re-administered without an immune reaction. This method is useful in clinical practice in order to tolerize the host to a

h f g e cg h e ec ec

useful recombinant adenovirus, and opens the possibility of providing effective long-term gene therapy for inherited metabolic diseases using these vectors.

Example 3

Oral Tolerization to Recombinant Adenovirus is Stable

[0184] To determine whether oral tolerization results in long-term tolerance, Gunn rats were tolerized by oral administration of adenoviral proteins as described in example 1. Eight months after the tolerization, the rats were injected with Ad-hBUGT.sub.1. This did not result in either humoral or CTL response. These results indicate that even in the absence of the presence of the tolerizing antigens, the tolerization is long-lived.

Example 4

Establishment of Oral Tolerization to Pre-Existing Immunoreaction

[0185] The previous examples have demonstated that long-term adenovirus-directed gene therapy, can be achieved by tolerizing the host specifically to antigens of the recombinant adenovirus by oral administration of adenoviral antigens. This permits long-term transgene expression without systemic immunosuppression. Some serotypes of adenoviruses, including adenovirus type 5, the serotype in which most gene therapy vectors have been constructed, commonly infect humans. Therefore, many adult humans have preexisting neutralizing antibodies and cytotoxic lymphocytes against adenoviruses, which would pose an obstacle to the clinical application of these vectors (Weiner (1994) supra; Vandenback (1994) supra).

[0186] The present example demonstrates that oral tolerization, in addition to preventing the appearance of host immune response, can also reduce preexisting antiadenoviral antibody titers and cytotoxic lymphocyte response. The results demonstrate, for the first time, that by enteral administration of the major adenoviral structural proteins into preimmunized rats it is possible to reduce the preexisting antiadenoviral immune response to a point at which it possible to express the transgene by intravenous injection of a recombinant adenovirus.

[0187] Details of the protocols for the tolerization viral administration and analysis of immunological effects were as described for Example 1. Details of the protocols for assessment of gene expression were as described for Example 2

[0188] Ad-hBUGT.sub.1 Injection into Gunn Rats:

[0189] Three groups of Gunn rats, two consisting of 10 animals in each (A and C), and one with five rats (B), were studied (Table 1). All rats were injected with the recombinant virus Ad hBUGT (5.times.10.sup.9 pfu/rat) and the induction of high titers of anti-adenovirus neutralizing antibodies was verified as described below. Groups A rats were fed with 10 doses of adenoviral-protein extracts (1 mg every other day) starting on day 40 after the first adenovirus injection. Rats in Group B were fed with 5 doses of the adenoviral proteins starting on day 10. Group C rats (control) received 10 doses of bovine serum albumin (1 mg every other day) starting on day 40. Rats in all groups received a second injection of Ad-hBUGT.sub.1 (5.times.10.sup.9 pfu) on day 72.

2TABLE 2 Experimental groups: Number Days of feeding (after Group (n) Antigen fed and dose of doses first virus injection) A 10 Adenoviral proteins 10 40-58 1 mg per rat daily B 5 Adenoviral proteins 5 10-18 1 mg per rat daily C 10 1 mg bovine serum 10 40-58 albumin per rat per day

h f g e cg h e ec ec

[0190] Assessment of Transgene Expression.

[0191] DNA Analysis Using PCR:

[0192] To detect the presence of the human BUGT.sub.1 gene in the host liver, DNA was extracted from RNase-treated tissue homogenates as described previouslyin Example 2. Two rats from each group were tested 5 days after the second Ad-hBUGT.sub.1 injection. DNA was subjected to amplification by polymerase chain reaction as described in Example 2

[0193] Expression of Human-BUGT.sub.1 Protein:

[0194] For determination of the expression of hBUGT.sub.1, liver specimens were taken from two rats in experimental Groups A and B, and control Group C five days after the second viral injection. Tissue homogenates were processed and analyzed as described in Example 2

[0195] Determination of Serum Bilirubin Levels:

[0196] Serum bilirubin levels were measured every 10-14 days throughout the study period as described in Example 2

[0197] Bile Pigment Analysis:

[0198] For definitive demonstration of bilirubin glucuronidation in selected rats, bile was collected and analyzed as described in Example 2. Bile was analyzed in two rats from each group, 14 days after the second injection.

[0199] Evaluation of Immune Tolerance.

[0200] Antiadenovirus Antibodies by Enzyme-Linked Immunosorbent Assay (ELISA):

[0201] Detection of anti-adenoviral antibodies by ELISA was performed by coating 96 well plates with 1.times.10.sup.8 particles per well of Ad-hBUGT in PBS at 4.degree. C. overnight. The wells were washed five times with 10 mM sodium phosphate containing 150 mM NaCl (PBS) and 1% Tween-20, blocked with 3% BSA in PBS, washed again and incubated for 2 hours with serial dilutions of the sera (in 1% BSA) at 37.degree. C. IgG antibody levels were measured after 0.1 M mercapthoethanol incubation of the sera for 1 hour at 37.degree. C. The wells were washed and incubated with 100 ml of 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rat IgG (Bethyl Laboratories, Montgomery, Tex.), for 2 hour at 37.degree. C. After washing, the wells were incubated with substrate (104 Phosphate Substrate, Sigma Diagnostics, St Louis), and read at 405 nm in an ELISA reader. Two negative control sera from naive Gunn rats, were included in each plate. End point titers were expressed as the reciprocal of the highest dilution that produced an absorbance at least two-fold greater than that observed with negative controls. Sera of rats from all groups were tested on days 0, 14, 70, 98 and 126, after the first injection.

[0202] Liver Histology:

[0203] For evaluation of the degree of hepatic inflammation, 10% formaldehyde-fixed liver biopsies were performed one week after the second injection in 2 rats from each group. Paraffin sections were stained with hematoxylin-eosin according to standard procedures. The sections were graded for hepatic inflammation as follows: Grade 0: normal; Grade 1: mild periportal or focal lobular lymphocytic

h f g e cg h e ec ec

infiltration; Grade 2: extension of lymphocytic infiltration into the lobules and "piece-meal necrosis"; and Grade 3: disruption of the lobular architecture by "bridging necrosis" and extension of lymphocytic infiltrates from portal to central, portal to portal and central to central zones.

[0204] Cytotoxic Lymphocyte Response:

[0205] Two rats from each group were studied on days 50 and 98. Spleens were removed under anesthesia from each of two rats at each time point, and the animals resutured. Subsequent analysis was as described in Example

[0206] Results

[0207] The Effect of Oral Tolerization.

[0208] Antiadenovirus Antibodies:

[0209] Serum IgG anti-adenovirus antibodies were examined by ELISA on days 0, 14, 70, 98 and 126 after the first injection, in all rats from groups A, B, and C. Anti-adenovirus antibodies appeared in all three groups after the first injection, with titers rising to a peak of 1:2.sup.10 at day 14 (FIG. 2). However after enteral administration of 10 doses of adenoviral antigens (Group A), the antibody titers decreased to 1:2.sup.7 at day 70 (FIG. 5, solid bar). In the BSA-treated controls (Group C), there was only a slight decrease (1:2.sup.9) in the anti-adenovirus antibody-titers (FIG. 5, open bars). Following the second injection of Ad-hBUGT.sub.1 on day 72 after the first injection, there was a boosting of antibody titers (1:2.sup.14) in the control group. In contrast, the antibody titers progressively decreased in the orally tolerized rats (Group A, FIG. 5, solid bars) despite the second injection of the recombinant adenovirus. Similar results were seen in the tolerized Group B, in which only 5 doses of the adenoviral proteins were administered between day 10 and 18 after the first adenovirus injection (not shown in FIG. 5). Twenty six and 54 days after the second adenovirus injection, the difference in antibody titers between the tolerized and non-tolerized groups were statistically significant ($p<0.005$, by Student's T test)

[0210] Cytotoxic Lymphocyte Response:

[0211] Cytotoxic T cells against rat hepatocytes infected with adenoviruses were tested twice (day 50 and day 98 after the first Ad-hBUGT.sub.1 injection) during the study, in two rats from Groups A and C. ALT activity released in the media from the hepatocyte targets was used to quantify the CTL response. ALT levels in the media were 449 and 409 IU in Group A, and 421 and 531 IU in Group C 50 days after the first virus injection. Twenty six days after oral tolerization, ALT activity released in the media decreased to 166 and 142 IU in Group A, even though these rats had received a second dose of Ad-hBUGT.sub.1 on day 72. In the BSA-treated controls (Group C) the CTL activity, as reflected by ALT activity in the media continued to be high (399 and 476 IU).

[0212] Liver Histology:

[0213] Liver biopsies from two rats in each group examined 24-72 h after the second injection showed minimal or no periportal or lobular lymphocytic infiltration in group A. In contrast, a severe inflammatory reaction (grade 3) was observed in liver specimens taken from group C (FIG. 6).

[0214] Expression of h-BUGT Gene After the Injection of Ad-hBUGT.sub.1 into Gunn Rats.

[0215] DNA Analysis Using PCR:

h f g e cg h e ec ec

[0216] Presence of human BUGT.sub.1 DNA in the liver of Gunn rats from groups A and B was tested by PCR after the second AdhBUGT.sub.1 injection. The expected 321-bp amplicon was seen only in rats from groups A and B, while control group C was negative. Normal human liver and liver from an untreated Gunn rat were used as positive and negative controls, respectively (FIG. 7).

[0217] Expression of Human-BUGT.sub.1 Protein:

[0218] Liver specimens were collected from two rats in groups A, B, and C, 5 days after the second Ad-hBUGT.sub.1 injection. Immunoreactive 52 kDa bands, corresponding to hBUGT.sub.1 were observed in the treated Gunn rats in groups A and B following the second injection, but not in rats from the control group C (FIG. 8). Normal human liver and untreated Gun rat livers were used as positive and negative controls respectively.

[0219] Serum Bilirubin Levels:

[0220] Bilirubin levels were measured every 10-14 days. A marked decrease in bilirubin levels occurred after each Ad-hBUGT.sub.1 injection in groups A and B, with levels reaching as low as 2.2, and 2.78 mg/dl after the first and second injections, respectively (FIG. 9). In contrast, in untolerized Gunn rats (Group C), the second Ad-hBUGT.sub.1 injection had no effect on serum bilirubin concentrations.

[0221] Bile Pigment Analysis:

[0222] HPLC analysis of bile collected from two rats in groups A, B and C, twenty days after the second injection of Ad-hBUGT.sub.1 showed excretion of bilirubin monoglucuronide and diglucuronide in the bile collected from group A and B. The two glucuronides accounted for more than 95% of the bile pigments, less than 5% being unconjugated bilirubin. This profile is similar to that seen in normal Wistar rats. In group C, bile pigment analysis following the second injection showed no significant conjugated bilirubin excretion detected in the bile.

[0223] This example demonstrates that the level of preexisting antibodies against adenoviruses can be suppressed by oral instillation of the major viral proteins into preimmunized rats. This procedure made it possible to readminister the virus with repeated rounds of transgene expression. The reduction of antibody titers occurs slowly because of the relatively long half-life of immunoglobulins, but importantly, the antibody levels continued to decrease in the tolerized group despite the second injection of Ad-hBUGT.sub.1. The findings indicate that the presence of antibodies in titers of up to 1:2.sup.7 does not impede gene transfer using adenoviral vectors. The lack of a detectable metabolic effect after the second administration of the virus in the non-tolerized (BSA-treated) group suggests that the strong secondary humoral or CTL response that resulted from the second injection may be responsible for attenuating the transgene expression by clearing the recombinant virus or virally infected hepatocytes.

Example 5

Oral Tolerization to Recombinant Adenovirus is Transferable

[0224] Although the preceding examples have demonstrated the induction of tolerance to recombinant Adenoviruses, there may be circumstances where the tolerization cannot be done in the subject. For instance it has previously shown that the presence of inmmunosuppressive drugs such as cyclosporin A may prevented the induction of oral tolerance (Fukushima, A. Whitcup, S. M., Nussenblatt, R. B., and Gery, I. "In Oral Tolerance; Mechanisms and Applications" (1996) 376-378, H. L. Weiner and L. F. Mayer (eds.) The New York Acadamy of Sciences, New York, N.Y., incorporated herein by reference).

h f g e cg h e ec ec

Presumably other circumstances that have shut down some of the immune system could also abrogate induction. Under such circumstances it may be useful to induce oral tolerization in one subject (donor) and then transfer this tolerance to a second subject (recipient).

[0225] Adoptive Transfer of Tolerance:

[0226] To determine whether the intestinal wall or splenic lymphocytes from the tolerized rats are capable of producing tolerance upon transplantation into naive rats, donor rats from groups A and C1 (2 rats from each group) from Examples 1 and 2 were killed at the end of the experiment and single suspensions of lymphocytes derived from the spleen or the small intestine were prepared as described previously for Cytotoxic T lymphocyte assays. The cells were resuspended in PBS immediately before transplantation. Recipients rats were sublethally irradiated with 600 rad total body irradiation, 24 hr prior to intravenous injection of 5.times.10.sup.7-1.times.10.sup.8 donor cells in 0.5 ml PBS. A total of eight rats were studied, four received the cells from group A donor rats, and four from group C rats (in each group, 2 rats received donor splenocytes, 5.times.10.sup.8 cells, and two received donor gut wall lymphocytes, 5.times.10.sup.7 cells). All rats were injected with Ad-hBUGT.sub.1 twice, one day after cell transplantation and 98 days later. Serum bilirubin levels, pigments excreted in bile and anti-adenoviral cellular and humoral immune responses were determined as described before.

[0227] Evaluation of Adoptive Transfer of Tolerance:

[0228] Levels of serum bilirubin in this example are shown in FIG. 10. Adoptive transfer of the tolerance was seen only in the two rats receiving the splenocytes from group A. Following AdhBUGT.sub.1 administration, these rats showed a metabolic effect similar to that observed in the tolerized rats from group A described in Example 2. Moreover, one of these rats did not develop anti adenovirus antibodies and the other mounted only a low titer antibody response. In contrast, when lymphocytes from untolerized donors or lymphocytes from the gut wall of tolerized donors were used, adoptive transfer of the tolerance did not occur and serum bilirubin returned to levels seen prior to administration of the recombinant adenovirus. Upon a second injection of the virus, these rats failed to show metabolic evidence of BUGT.sub.1 expression. In the recipients of cells from group C rats, all the rats developed a marked anti-adenoviral humoral and cellular immune response, and all lost the transgene effect within 6 weeks of the injection.

[0229] This example demonstrates that the induction of oral tolerance in one subject is a state that can be transferred into a second subject.

Example 6

Oral Tolerization to Recombinant Adenovirus in Rabbits and Demonstration That Tolerance is Also Established to a Non-Native Transgene

[0230] In the previous examples, oral tolerization has provided a stable expression of a recombinant Adenovirus and the tolerization was sufficient to enable expression after a readministration of this Adenovirus vector. However, these examples were performed using rats as subjects and the gene being expressed (BUGT.sub.1) although derived from a human source may be sufficiently similar to the the native product that there may not have been an elicitation of an immune reaction to the transgene. The absence of BUGT.sub.1 gene expression after the second administration may have been a reaction to the vector alone. The following example differs from the previous example in that rabbits are used as the subjects and the gene used in the first and second recombinant Adenovirus administrations is lacZ which is derived from E. coli.

h f g e cg h e ec ec

[0231] Details of the methods used for induction and evaluation of oral tolerance are as described for the previous examples with the Gunn rats with the following exceptions:

[0232] a) the rabbits were fed antigens orally rather than using intubation

[0233] b) Due to their larger size, the amount of antigen used for tolerization was increased proportionally (10 mg instead of 1 mg)

[0234] Evaluation of Oral Tolerance;

[0235] FIG. 11 shows that there is a high level of expression of the lacZ gene after introduction into rabbit hepatocytes by adenovirus As seen in the rat system, this is a transient effect and most activity has disappeared after three weeks (FIG. 12). When the rabbit is tolerized by oral administration of the Ad-hBUGT.sub.1 vector, a second injection with the recombinant Adenovirus allows efficient expression of the lacZ gene (FIG. 13). On the other hand, without this tolerization, a second injection is incapable of expressing any significant levels of b-galactosidase activity (FIG. 14). This example demonstrates that the present invention is not restricted to rats alone and works efficaciously in other animals. Furthermore, the high level of expression of lacZ after the second administration of recombinant adenovirus demonstrated that there was tolerization to an enzyme that is not native to the subject.

Example 7

Example: Delivery of Protein Molecules to Cells

[0236] Bovine Serum Albumin was labeled by conjugation with both biotin and fluorescein (BSA-BF) or with fluourescein alone (BSA-F) by the following method:

[0237] BSA-BF: BSA (68 mg) was dissolved in 4.8 ml of 0.2M borate buffer, pH 9.0. Biotin-21-NHS ester (4 umoles) in 2 ml of DMF was added dropwise, and the mixture was incubated at room temperature for 2 hours. Fluorescein isothiocyanate (2 umoles) in 200 ul DMF was added and the mixture was incubated overnight at room temperature. The mixture was evaporated to dryness in a rotary evaporator and then redissolved in 5 ml H.sub.2O and applied to a G-25 column equilibrated with 50 mM Tris buffer, pH 8.0. The fluoresceinated fractions were collected and stored at 0.degree. C.

[0238] BSA-F: This was prepared by the above procedure except that the reaction with biotin 21-NHS was omitted.

[0239] U937 cells were grown to approximately 10.sup.6/ml and centrifuged, washed in growth medium (RPMI), suspended in 3.5 ml of RPMI, and 0.8 ml was placed in each of four 35 mm wells. BSA-BF and BSA-F, each at a concentration of 20 mg/ml, were added to the four wells together with 200 ul of RPMI as follows:

[0240] 1) 20 ul BSA-F

[0241] 2) 50 ul BSA-F

[0242] 3) 20 ul BSA-BF

[0243] 4) 50 ul BSA-BF.

[0244] The cell suspensions were incubated overnight at 37.degree. C. One ml of cell suspension was

h f g e cg h e ec ec

placed in 2.0 ml of RPMI and centrifuged at 1000 rpm for 5 minutes. The supernatants were decanted and the cells were resuspended in 200 ul of RPMI. Samples of each cell suspension were placed on a microscope slide and examined using a fluorescent microscope. The following was observed:

[0245] 1) 20 ul BSA-F: Against a slight background of fluorescense, fewer than 5% of the cells displayed any fluorescense,

[0246] 2) 50 ul BSA-F: Against a slight background of fluorescense, fewer than 5% of the cells displayed any fluorescense,

[0247] 3) 20 ul BSA-BF: Approximately 40% of the cells displayed an intense fluorescense,

[0248] 4) 50 ul BSA-BF: Approximately 40% of the cells displayed an intense fluorescense.

[0249] Evidence that the biotinylated BSA entered cells was provided by observations of cells that were undergoing mitosis as indicated by the presence of two nuclei in a single cell. Such cells displayed a bright fluorescense that was confined to the nuclei.

Example 8

TGF-b.sub.1 Levels as a Marker for Oral Tolerization

[0250] The following example demonstrates the method for establishing that tolerization has been conferred by measuring TGF-b.sub.1 levels The samples were derived from the rats described in Examples 1 and 2.

[0251] Serum TGF-b1 Levels.

[0252] TGF-b1 levels were measured by a "sandwich" ELISA using Genzyme Diagnostics kit according to manufacturers' instructions. Serum TGF-b1 levels were measured in three rats from each group after each injection, on days 8 and 101.

[0253] Measurement of TGF-b 1 Secreted by Intestinal Lymphocytes and Splenocytes:

[0254] For the extraction of gut wall lymphocytes, the small intestines were removed from 2 rats each from the tolerized and the control groups on day 101, and placed in RPMI medium supplemented with 15% FBS. The intestines were cuts into 1 cm segments, flushed with the medium, opened by cutting longitudinally and transferred into fresh medium, rinsed four times with PBS and placed in PBS (calcium and magnesium free), containing 1 mM EDTA and 1 mM dithiothretol (DTT). Fragments were stirred for 30 minutes at 37.degree. C. and the exfoliated cells were harvested by decanting the PBS after tissue fragments had settled. Cells were passed through nylon wool, pelleted by centrifugation for 5 minutes, suspended in 10 ml of 40% Percoll and layered over a cushion of 70% Percoll. Cells were then centrifuged for 20 min at 600.times.g and the gut wall lymphocytes at the 70%-40% interface were harvested.

[0255] For enrichment of antigen presenting cells, lymphocytes were harvested from the spleens as described above. Cells were then suspended in 4 ml of RPMI containing 5% fetal calf serum (FBS) and 4 ml of RPMI containing 5% FBS and 14.5 g% metrizamide. After centrifugation at 1800.times.g for 20 min, at room temperature, the interface containing an enriched population of macrophages and dendritic cells was collected.

h f g e cg h e ec ec

[0256] For determination of TGF-b1 secretion, intestinal wall or splenic lymphocytes from untreated Gunn rats, rats from the control groups C and D, and rats from the tolerized group A after each injection, on days 8 and 101 (two rats from each group) were plated on tissue culture dishes (5.times.10.sup.8/10 cm plate) and grown in serum-free media. 1 .times.10.sup.6 antigen presenting cells were added per plate along with 50 mg of adenoviral protein extracts as the activating antigen. After 72 hr of culturing, TGF-b1 secreted into the media was quantified by ELISA as described above.

[0257] Measurements of Serum TGFb.sub.1 Concentrations and TGFb.sub.1 Secretion In Vitro:

[0258] Serum TGFb.sub.1 levels were increased to >170 ng/ml after each injection of the recombinant adenovirus in rats that were given the 1 mg/day dose of adenoviral proteins before the injection of recombinant viruses (group A). In rats that received BSA or no protein before the virus injection (Groups C and D) serum TGFb.sub.1 levels were 30-35 ng/ml ($p<0.005$). The levels in normal untreated Gunn rats are 18-26 ng/ml.

[0259] In vitro assays for evaluation of TGFb.sub.1 levels after exposure of splenocytes and gut wall lymphocytes to adenoviral antigens were done in two rats from each group after each virus injection. Low levels (>4 ng/ml) of TGFb.sub.1 were present in the supernatant of splenocytes and gut wall lymphocytes cultures from the rats that were administered BSA or no protein (group C and D); silimar levels of TGFb.sub.1 were observed using gut wall lymphocytes from tolerized rats (normal levels: 2.1-3.6 ng/ml). In contrast, splenocytes from rats tolerized by feeding adenoviral proteins (group A) secreted significantly greater amounts of TGFb.sub.1 (24-26 ng/ml) after exposure to viral proteins.

Example 9

Assessment of Various Cytokines as Markers for Oral Tolerization

[0260] The following example demonstrates the method for establishing that tolerization has been conferred by measuring the levels of various cytokines The samples were derived from the rats described in Examples 1 and 2

[0261] RT-PCR for Rat Cytokine mRNAs:

[0262] These assays were performed on day 101 on two rats from each of groups A, C, D and E. After culturing the lymphocytes with the viral antigens and antigen presenting cells as described above, cells were harvested and mRNA levels for rat IL-2, 4, 6, 10, IFN-g and TGF-b1 were determined by reverse transcription-primed polymerase chain reaction (RT-PCR One mg of RNA was used as template for each sample. Amplimers for rat glyceraldehyde-3-phosphate dehydrogenase (GPDH) were used as an internal control for the RT-PCR.

[0263] Results of RT-PCR for Rat Cytokines:

[0264] RT-PCR was performed on days 8 and 101 on RNA extracted from and gut wall lymphocyte cell cultures from the various groups. Positive bands for IL-2, 4, 10, and TGFb1 were found in splenocytes from rats tolerized by adenoviral protein administration (group A), but not from rats that had received BSA or no protein at all (groups C or D). Gut wall lymphocytes cell cultures from the tolerized rats (group A), as well as from rats that received BSA or no proteins (groups C or D), were negative for these cytokine mRNAs. In contrast, IFNg was negative by RT-PCR in splenocytes from the tolerized rats in group A, but was found in splenocytes from rats from control groups C and D. Gut wall lymphocytes (GW) showed similar results to non-tolerized rats. IL6 was detected in all tested groups and probably behaves as a non specific acute phase reactant

h f g e cg h e ec ec

[0265] Many obvious variations will be suggested to those of ordinary skill in the art in light of the above detailed description and examples of the present invention. All such variations are fully embraced by the scope and spirit of the invention as defined by the claims that follow.

* * * * *

h f g e cg h e ec ec

CROATIAN MEDICAL JOURNAL
CMJ
42(4):446-450,2001
BASIC SCIENCES

Liver Cell Transplantation – Novel Animal Model for Human Hepatic Viral Infections

Catherine H. Wu, Edwin C. Ouyang, Cherie Walton, George Y. Wu

Department of Medicine, Division of Castroenterology-Hepatology, University of Connecticut Health Center, Farmington, Conn, USA

Aim. To generate a model of human hepatitis B infection (HBV) in immunocompetent rats with chimeric human liver.

Results. Normal rats were tolerized to human hepatocytes by exposure to human hepatocytes at day 17 of gestation. We transplanted human hepatocytes and inoculated HBV into the rats after birth. Mixed lymphocytes assay, a measure for tolerance, indicated that animals fetally exposed to human hepatocytes developed tolerance to human hepatocytes. Spleen lymphocytes from tolerized animals did not proliferate when challenged with donor human hepatocytes. In contrast, control animals given saline fetally developed no tolerance to human hepatocytes. Tolerant animals with transplanted human hepatocytes were susceptible to HBV infection. Western blot analysis and immuno-histochemistry of liver sections from tolerized, HBV infected animals with transplanted human hepatocytes showed the presence of functioning human hepatocytes that synthesized human albumin, of which 30% were also positive for HB surface antigen and HBV DNA. The presence of covalent closed circular HBV DNA in the liver indicated active HBV viral replication.

Conclusion. Tolerized rats with chimeric human livers can be infected with HBV and used as an animal model for HBV infection. Tolerized rats with chimeric human livers can also be used for generating models of other human hepatic viral diseases.

Key words: *cell transplantation; hepatitis B surface antigens; hepatitis B virus; liver; models, animal; transplantation tolerance*

The study of many human diseases is hampered by the lack of useful animal models. This is especially true for viral infections of the liver that result in acute and chronic hepatitis. Persistent hepatitis B (HBV) (1) and chronic hepatitis C (HCV) (2) viral infections are major health problems worldwide. HBV and HCV are highly tissue- (liver) and species-selective. HBV animal models based on homologous viruses in non-primates, such as woodchuck (3), or HBV in immunodeficient (4) or transgenic animals (5), while providing useful information lack many aspects of human disease. Beside the chimpanzee, there are no animal models for HCV (6). Research on transgenic animals expressing individual HCV gene products has yielded conflicting results where the function of the proteins is concerned. Until there is an experimental model system that supports HCV replication and infection, the role of each individual protein in the pathogenic outcome of HCV infection can not be clearly delineated.

In recent years, transplantation of normal or genetically altered syngeneic and allogeneic hepatocytes has been used successfully for gene therapy of different diseases in animal models (7,8) and humans (9,10). Hepatocyte transplantation experiments have also provided useful information on the localization and vascular and tissue requirements for the maintenance of transplanted cells (11), liver gene regulation (12), and regulation of liver growth. In most allogeneic transplantation experiments, immunosuppression has been necessary to sustain functioning transplanted cells. In developing animal models for human HBV or HCV viral diseases, it is important that they have intact and normal immune system because the host immune system plays an important role in the pathogenesis of the viral infections (13). Our hypothesis was that tolerizing rats with immature immune system to human hepatocytes would result in rats that can host transplanted human hepatocytes. Tolerant rats with chimeric human livers could be infected with HBV or HCV. Here we review our data that supported this hypothesis.

Generation of Chimeric Human Liver in Normal Rats

Induction of Tolerance to Human Hepatocytes

The major obstacle in establishing chimeric human liver in a normal rat is the rejection of transplanted xenogenic human cells by the host immune system. Therefore, our first step was to induce donor-specific tolerance towards human cells in the host before the transplantation.

Earlier, Medawar and co-workers (14) showed that "actively acquired tolerance" to foreign cells could be achieved by exposing fetal animals to the foreign cell. This method of tolerization was used by Kline et al (15) for significant prolongation of cardiac allograft survival. To induce tolerance, human hepatocytes were injected into intraperitoneal cavity of fetal rats at 15-17 days of gestation, a time frame when T-cells are educated to distinguish self from foreign antigens. Mixed lymphocyte assay, a measure of tolerance, indicated that spleen lymphocytes from control saline-treated animals were stimulated to proliferate when mixed with human hepatocytes, as indicated by the increased ^{3}[H]-thymidine uptake (Fig. 1, column 3). In contrast, lymphocytes from the animals that were fetally exposed to human hepatocytes were not stimulated to proliferate when mixed with donor human hepatocytes (Fig. 1, column 4). This indicated that the animals became tolerant to human hepatocytes. Similarly, lymphocytes from the animals that were fetally exposed to and subsequently transplanted with human hepatocytes were not stimulated to proliferate when mixed with donor human hepatocytes (Fig. 1, column 5). The degree of ^{3}[H]-thymidine uptake in lymphocytes from fetally tolerized rats when exposed to donor human hepatocytes was similar to that found in lymphocytes alone (Fig. 1, column 1) or in human hepatocytes alone (Fig. 1, column 2).

In animals, intrathymic injection of the donor cells or antigens (16) or oral gavaging of large concentrations of foreign antigen (17) can also induce tolerance to foreign antigen and donor cells. We tested which of the three methods would be optimal in inducing tolerance to human hepatocytes in the normal rat (Fig. 2). Although intrathymic injection (Fig. 2, column 3) and oral ingestion (Fig. 2, column 4) can induce tolerance, exposure of fetal rats to human hepatocytes (Fig. 2, column 2) produced the greatest degree of tolerance. Thus, rats with normal immune system can be made tolerant to human hepatocytes by fetal exposure to the human cells.

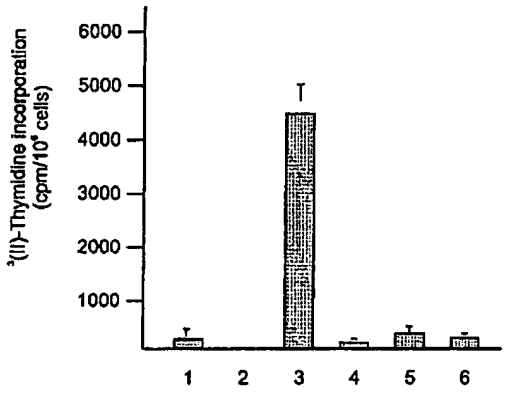

Figure 1. Mixed lymphocyte assay for tolerance. Responder cells: rat spleen lymphocytes. Stimulator cells: -radiation-inactivated donor human hepatocytes. Column 1: spleen lymphocytes from rat with fetal saline injection. Column 2: spleen lymphocytes from saline treated rat stimulated with irradiated lymphocytes from the same animal. Column 3: spleen lymphocytes from saline treated rat stimulated with irradiated donor human hepatocytes. Column 4: spleen lymphocytes from rat with fetal exposure to human hepatocytes and stimulated with irradiated donor human hepatocytes. Column 5: spleen lymphocytes from rat with fetal exposure and transplanted human hepatocytes, and stimulated with irradiated donor human hepatocytes. Column 6: irradiated donor human hepatocytes alone.

Transplantation of Human Hepatocytes into Tolerized Rats

Can rats tolerant to human hepatocytes be successful hosts to transplanted human hepatocytes?

To answer this question, human hepatocytes were transplanted to rats fetally tolerized with human hepatocytes at 17 day of gestation within 24 h after their birth. To determine the success of human hepatocyte transplantation, serum and liver samples were collected. Western blot analysis using an affinity purified anti-human albumin antibody indicated that human albumin could be detected in the serum of tolerized rats that received transplanted hepatocytes (Fig. 3). The antibody was specific for human albumin (Fig. 3, column 1) and could not detect standard rat albumin (Fig. 3, column 2). In tolerized rats that received transplanted column hepatocytes, human albumin could be detected in serum at least up to 6 weeks after transplantation (Fig. 3, columns 3-7). Rats tolerized and transplanted with a human fibroblast cell line, IMR 90, did not produce human serum albumin (Fig. 3, column 8), which indicated that albumin production is specific to human liver cells only.

Human albumin production by transplanted human liver cells was also confirmed by immunofluorescence of fresh frozen liver tissues. Figure 4 is a representative immunofluorescence of liver section taken from rat 3 weeks after the transplantation. In control animals, which fetally received injection of saline and

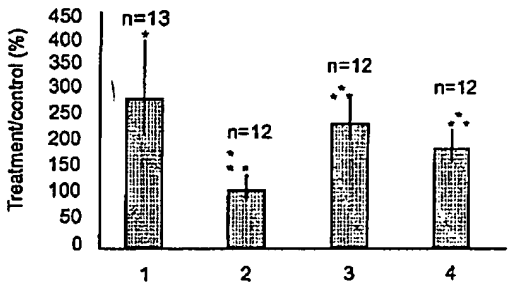

Figure 2. Mixed lymphocyte assay of spleen lymphocytes from fetal, intra-thymic and oral tolerization. Column 1: spleen lymphocytes cells from non-tolerized, saline-treated control rat stimulated with irradiated human hepatocytes. Column 2: spleen lymphocytes from fetally tolerized rat stimulated with irradiated donor human hepatocytes. Column 3: spleen lymphocytes from rat after intra-thymic tolerization, stimulated with irradiated donor human hepatocytes. Column 4: spleen lymphocytes from orally tolerized animal stimulated with irradiated donor human hepatocytes. N – number of rats. One asterisk indicates $p < 0.05$ between column 1 and columns 2, 3, or 4. Two asterisks indicates $p < 0.05$ between column 2 and columns 3 and 4.

were not transplanted with human hepatocytes, no immunofluorescence for human albumin could be detected (Fig. 4, panel A). Three weeks after human hepatocytes transplantation, tolerized rat was positive for human albumin, as indicated by the positive fluorescent cells (Fig. 4, panel B), whereas tolerized control animals were not positive for human albumin (Fig. 4, panel C).

Generation of a Rat Model of HBV Infection in Tolerized Rats with Chimeric Human Liver

To generate HBV infection, tolerized rats to which human hepatocytes were transplanted 24 h after birth were inoculated with HBV virus one week after the transplantation. Serum samples were collected weekly. Serial liver biopsies were performed at timed intervals after infection. Immuno-histochemical analysis (Fig. 5) revealed that human hepatocytes containing human albumin and producing HBV surface antigen were present in rat livers at 15 weeks after HBV infection (Fig. 5, panels A and B). In addition, 30% of the cells positive for human albumin were also positive for HBV surface antigen. Livers from tolerized animals that received transplanted hepatocytes but had no HBV infection were positive for human albumin (Fig. 5, panel C), but did not have HBV surface antigen staining (Fig. 5, panel D). When inoculated with HBV, these tolerized animals were negative for both human albumin and HBV surface antigen (Fig. 5, panels E and F). To determine that HBV surface antigen in the human liver cells did indeed come from viruses, the presence of HBV viral DNA (HBV DNA) was detected by *in situ* hybridization with a digoxigenin-HBV DNA probe (Fig. 6). HBV DNA positive cells were present only in the liver from tolerized animals that received transplanted hepatocytes and were infected with HBV (Fig. 6, panel A). Animals that did not receive transplanted human hepatocytes but were infected with HBV were not positive for HBV-DNA (Fig. 6, panel C). Similarly, livers from tolerized control animals (Fig. 6, panel D) and animals tolerized

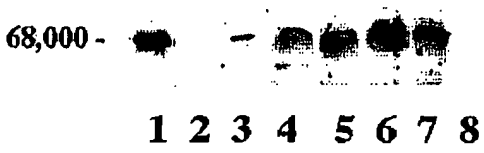

Figure 3. Western blot of rat serum, developed with anti-human albumin antibody and horseradish peroxidase-labeled secondary antibody. Lane 1: 10 ng standard human albumin. Lane 2: 10 ng standard rat albumin. Lane 3-7: serum from tolerized rat with transplanted human hepatocytes. Lane 3: two days after transplantation. Lane 4: two weeks after transplantation. Lane 5: three weeks after transplantation. Lane 6: five weeks after transplantation. Lane 7: six weeks after transplantation. Lane 8: serum from a rat tolerized and transplanted with human fibroblast cell line IMR-90.

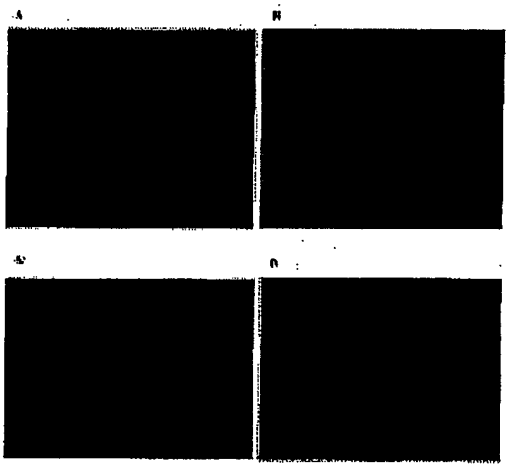

Figure 4. Immunofluorescence of fresh frozen liver tissue using anti-human antibody and Texas-red labeled secondary antibody. **Panel A:** liver from control fetally treated rat without transplanted human hepatocytes. **Panel B:** liver from fetaliy tolerized rat 3 weeks after human hepatocytes transplantation. **Panel C:** liver from fetally tolerized rat without transplanted cells. **Panel D:** same as panel B, but without secondary antibody.

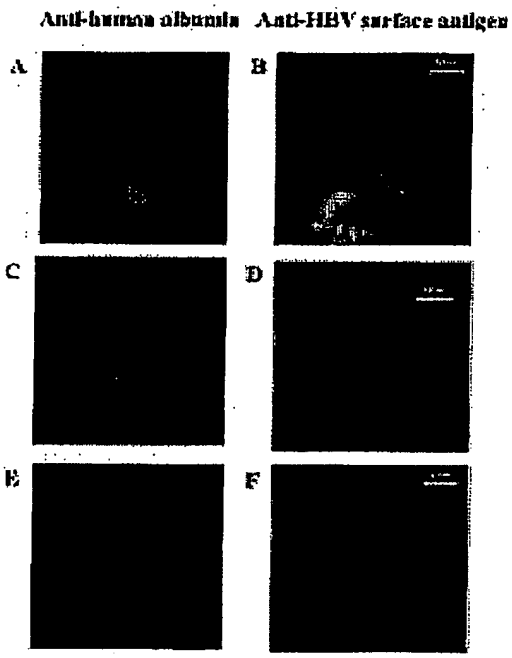

Figure 5. Immunofluorescence of fresh frozen liver tissue using anti-human antibody developed with Texas-red labeled secondary antibody and anti-HBV surface antigen developed with FITC-labeled secondary antibody. **Panels A and B:** liver from tolerized rat with transplanted human hepatocytes, and infected with HBV at 15 weeks after infection. **Panels C and D:** liver from tolerized rat with transplanted human hepatocytes but without HBV infection. **Panels E and F:** liver from tolerized rat without transplanted human hepatocytes but inoculated with HBV.

with human liver cell transplantation (Fig. 6, panel B) were negative for HBV DNA. If infection had taken place and viruses were replicating, DNA from the viruses should be present in sera of infected animals. Serum HBV DNA was confirmed by the presence of an expected 355-bp fragment of HBV genome spanning nt 2079-2434 (Fig. 7). Serum HBV DNA was detectable at 1 week after the infection (Fig. 7, lanes 4 and 7) and remained detectable over 15 weeks (the duration of the experiment) after HBV inoculation (Fig. 7, lanes 6 and 9). Serum HBV DNA was not detected in tolerized rats that received transplanted hepatocytes and were not inoculated with HBV (Fig. 7, lanes 10 and 11), nor was it found in tolerized rats that did not receive transplanted hepatocytes but were inoculated with HBV (lanes 12-17). At 15 weeks after HBV infection, there were 5,000 copies of HBV genome/mL serum in tolerized rats transplanted with human liver cells and inoculated with HBV. Finally,

Figure 6. *In situ* hybridization (digoxigenin-HBV DNA probe) of frozen liver sections for HBV DNA, detected with diaminobenzedine. **Panel A:** liver from a tolerized rat that received transplanted human hepatocytes and was infected with HBV. **Panel B:** liver from a tolerized rat that received transplanted human hepatocytes but was not infected with HBV. **Panel C:** liver from a tolerized rat without transplantation but inoculated with HBV. **Panel D:** liver from a tolerized rat without transplantation or HBV inoculation. (Permission to reprint this figure was obtained from Blackwell Publishers/ Blackwell Science Ltd/ Polity Press, Osney Mead, Oxford OX2 OEL, UK.)

Figure 7. PCR detection of serum HBV DNA. Lane 1: DNA molecular weight markers. Lane 2: serum from control untreated rat. Lane 3: positive control DNA from HBV producing HepG2.2.15 cells. Lanes 4-6 and 7-9: serum from two representative tolerized rats that received transplanted human hepatocytes and were inoculated with HBV, at 1, 5, and 15 weeks, respectively, after inoculation. Lanes 10-11: serum from tolerized rats that received transplanted human hepatocytes but were not inoculated with HBV. Lanes 12-14 and 15-17: serum from tolerized rats that did not receive transplantated human hepatocytes but were inoculated with HBV, at 1, 5, and 15 weeks, respectively, after inoculation. (Permission to reprint this figure was obtained from Blackwell Publishers/ Blackwell Science Ltd/ Polity Press, Osney Mead, Oxford OX2 OEL, UK.)

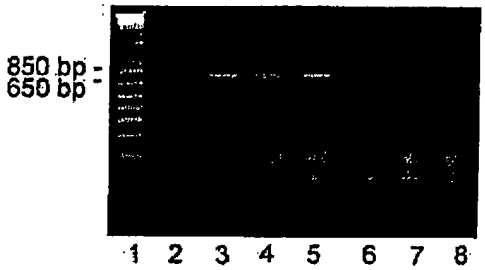

Figure 8. PCR detection of covalent closed circular HBV DNA in liver tissues. Lane 1: DNA molecular weight markers. Lane 2: control untreated rat. Lane 3: DNA from HBV producing HepG2.2.2.15 cells. Lanes 4 and 5: DNA from two tolerized rats transplanted with human hepatocytes and inoculated with HBV at 15 weeks after inoculation. Lane 6: liver from tolerized rat with human hepatocytes transplantation but without HBV inoculation at 15 weeks. Lanes 7 and 8: livers from two tolerized rats without transplantation but with HBV inoculation at 15 weeks after inoculation.

HBV replication could be demonstrated in livers by the presence of covalent closed circular HBV DNA (Fig. 8). It was detected by polymerase chain reaction primers specific to the region of HBV genome that is incomplete in the plus strand of the viral particle, but is covalently closed during HBV replication (Fig. 8, lanes 4 and 5). The same 698-bp band of cDNA could not be detected in livers of tolerized animals that received transplanted cells but were not infected by virus (Fig. 8, lanes 7 and 8) or in livers of tolerized animals that did not receive the transplant but were infected with HBV (Fig. 8, lane 6).

Conclusion

Rats that were fetally tolerized to human hepatocytes could host human hepatocytes in the liver and were susceptible to HBV infection. Tolerized animals with chimeric human livers could also be used to develop a rat model for HCV.

Acknowledgments

This work was partly supported by grants from Connecticut Innovations, Inc. (CHW), the Herman Lopata Chair in Hepatitis Research (GYW), and NIDDK: DK-42182 (GYW).

Permission to reprint Figures 6 and 7 from the original article: Wu CH, Ouyang EC, Walton CM, Wu GY. Human hepatocytes transplanted into genetically immunocompetent rats are susceptible to infection by hepatitis B virus in situ. J Viral Hepat 2001;8:111-9. Published by Blackwell Publishers/ Blackwell Science Ltd/ Polity Press, Osney Mead, Oxford OX2 OEL, UK.

References

1 Gerety R, editor. Hepatitis B. New York (NY): Academic Press; 1985.

2 Fishman JA, Rubin RH, Koziel MJ, Periera BJ. Hepatitis C virus and organ transplantation. Transplantation 1996; 62:147-54.

3 Rogler CE, Summers J. Cloning and structural analysis of integrated woodchuck hepatitis virus sequences from a chronically infected liver. J Virol 1984;50:832-7.

4 Petersen J, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogenic hepatocytes in B and T cell deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc Natl Acad Sci USA 1998;95:310-5.

5 Farza H, Hadchouel M, Scotto J, Tiollais P, Babinet C, Pourcel C. Replication and gene expression of hepatitis B virus in a transgenic mouse model that contains the complete viral genome. J Virol 1988;62:4144-52.

6 Grakoui A, Hanson HL, Rice CM. Bad time for Bonzo? Experimental models of Hepatitis C virus infection, replication and pathogenesis. Hepatology 2001;33:489-95.

7 Ott M, Schmidt HHJ, Cichon G, Manns MP. Emerging therapies in hepatology: liver-directed gene transfer and hepatocyte transplantation. Cell Tissue Organs 2000: 167:81-7.

8 Gupta S, Rogler CE. Lessons From Genetically Engineered Animal Models VI. Liver repopulation systems and study of pathophysiological mechanisms in animals. Am J Physiol 1999;277(6 Pt 1):G1097-102.

9 Strom SC, Chowdhury JR, Fox IJ. Hepatocyte transplantation for the treatment of human disease. Semin Liver Dis 1999;19:39-48.

10 Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Eng J Med 1998;338:1422-6.

11 Gupta S, Rajvanshi P, Sokhi R, Slehria S, Yam A, Kerr A, Novikoff PM. Entry and integration of transplanted hepatocytes in liver plates occur by disruption of hepatic sinusoidal endothelium. Hepatology 1999;29: 509-19.

12 Gupta S, Rajvanshi, Sokhi R, Vaidya S, Irani AN, Gorla GR. Position-specific gene expression in the liver lobule is directed by the microenvironment and not by the previous cell differentiation state. J Biol Chem 1999; 274:2157-65.

13 Rehermann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. J Clin Invest 1996;98:1432-40.

14 Billingham RE, Brent L, Medawar PB. 'Actively acquired tolerance' of foreign cells. Nature 1953;172: 603-6.

15 Kline GM, Shen Z, Mohiuddin M, Ruggiero V, Rostami S, DiSesa VJ. Development of tolerance to experimental cardiac allograft in utero. Ann Thorac Surg 1994;57: 72-5.

16 Ilan Y, Attavar P, Takahashi M, Davidson A, Horwitz MS, Guida J, et al. Induction of central tolerance by intrathymic inoculation of adenoviral antigens into the host thymus permits long-term gene therapy in Gunn rats. J Clin Invest 1996;98:2640-7.

17 Takahashi M, Ilan Y, Chowdhury NR, Guida J, Horwitz M, Chowdhury JR. Long term correction of bilirubin-UDP-glucuronosyltransferase deficiency in Gunn rats by administration of recombinant adenovirus during the neonatal period. J Biol Chem 1996;271: 26536-42.

Received: May 31, 2001
Accepted: June 28, 2001

Correspondence to:
Catherine H. Wu
Department of Medicine
Division of Gastroenterology-Hepatology
University of Connecticut Health Center
Rm. AM-045
263 Farmington Avenue
Farmington, CT 06030-1845, USA
cwu@nso1.uchc.edu

A NOVEL IMMUNOCOMPETENT RAT MODEL OF HCV INFECTION AND HEPATITIS

Short Title: HCV hepatitis in rats

George Y. Wu, Masayoshi Konishi, Cherie M. Walton, Denise Olive,

Kazuhiko Hayashi, and Catherine H. Wu

Department of Medicine, Division of Gastroenterology-Hepatology,

University of Connecticut Health Center, Farmington, CT

This work was supported in part by grants from American Liver Foundation/Blowitz Ridgeway Foundation [CHW], NIDDK: DK-42182 [GYW], the Herman Lopata Chair in Hepatitis Research [GYW], and Hepaticus, Inc. [CHW & GYW].

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; HBV, hepatitis B virus; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction

Address correspondence to:

George Y. Wu, M.D., Ph.D.

Department of Medicine

Division of Gastroenterology-Hepatology, Rm. AM-044

263 Farmington Avenue

Farmington, CT 06030-1845

(860) 679-3158

(860) 679-3159 (FAX)

wu@nso.uchc.edu

Abstract

Background & Aims: Hepatitis C virus (HCV) infects millions of people worldwide. Effectiveness of therapy depends on many variables, but in the US, treatment does not produce a sustained response in the majority of patients. Development of new agents has been hampered by the lack of a convenient animal model. Our objective was to determine whether an immunocompetent rat, tolerized and transplanted with a human hepatoma cell line (Huh 7 cells), could be used to sustain an HCV infection. *Methods:* For tolerization, fetal rats were injected *in utero* with 10^5 Huh 7 cells. One day after birth, rats were transplanted with 5×10^6 Huh 7 cells, and a week later inoculated with HCV, genotype 1. *Results:* In tolerized, transplanted and HCV-infected rats, Huh 7 cells in the liver were found by fluorescent antibodies against human albumin, and HCV viral replication by the presence of negative strand HCV RNA. HCV levels in serum by real time PCR were measured at 11,000 copies/ml at week 4, peaked at 22,500 copies/ml by week 16. In tolerized, transplanted, inoculated rats, but not controls, serum alanine aminotransferase (ALT) values rose to 60 IU/L by week 4, and reached a peak of about 120 IU/L by week 13. Histology showed foci of mononuclear infiltrates in portal and central regions. *Conclusions:* HCV-inoculated immunocompetent rats tolerized and transplanted with Huh 7 cells support HCV gene expression, viral replication, and develop biochemical and histological evidence of hepatitis.

Background & Aim

Hepatitis C infection represents a serious health problem worldwide, and a formidable challenge for therapy. One of the major impediments toward rapid progress in the field has been a lack of convenient animal models that mimic human HCV hepatitis. There are two major reasons for the difficulty in establishing animal models for HCV infection. First, the infection is species selective for humans, chimpanzees, and a few other primates.[1] Second, although there is some evidence that certain genotypes have cytotoxicity[2], the majority of the damage occurs as a result of the host immune response to the infection. Thus, the development of a non-primate, rodent model with a normal immune system could be of value in the study of viral infection and host reaction to it. Based on these principles, one strategy to produce an HCV infection in a non-primate would be to use human hepatocytes as host cells. Our objective was to develop an immunocompetent rat as the non-primate host. However, in the presence of a normal immune system, human cells would be recognized as foreign, and would be rejected. To circumvent this problem, advantage was taken of the fact that the rat immune system does not develop until 15-17 days of gestation.[3] We have shown previously that if human hepatocytes were introduced before or during this period, the immune system could be manipulated into recognizing human hepatocytes as "self" and not reject them.[4,5] The goal of the current study was to determine whether a human hepatoma cell line transplanted into immunocompetent rats would be susceptible to an HCV infection.

MATERIALS AND METHODS

Cells

Huh 7 cells, a human hepatoblastoma cell line, were maintained in Dulbecco Modified Eagle's Medium (Gibco/BRL) containing 10% heat inactivated fetal bovine serum (Gibco/BRL) at 37^0C in 6% CO_2. For injection into animals, trypsinized cells were washed extensively in sterile phosphate buffer saline (PBS), pH 7.0, and 1 x 10^7 cells were suspended in 1 ml PBS for use in animals.

Animals

Animal handling and surgery were performed in accordance with protocols approved by the University of Connecticut's Animal Care and Use Committee, and conformed to USDA and NIH animal usage guidelines. Sprague-Dawley female rats, 15 days timed-pregnant, were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), maintained on 12 hour light-dark cycles, and fed *ad lib* with standard rat chow.

Human materials

The use of HCV-positive human serum for inoculation, and human liver biopsy samples in this study were done in accordance with protocol #00-291 approved by the University of Connecticut Institutional Review Board.

Tolerization to a human hepatoma cell line

At 17 days of gestation, pregnant rats were anesthesized with ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively), and the abdominal cavity and gravid uterus were exposed by

laparotomy under aseptic conditions. Huh 7 cells, 1 x 10^5, in 10 µl PBS were injected through the uterine wall into intraperitoneal cavity of fetal rats as described by us previously.[4]

Transplantation of Huh 7 cells and inoculation with HCV

Twenty four hours after birth, groups of tolerized rats were transplanted with Huh 7 cells by slowly injecting 5 x 10^6 cells in 100 µl PBS directly into the spleen through the translucent abdominal skin wall. One week after transplantation, tolerized rats were inoculated with 10^5 copies HCV (genotype 1) in 100 µl human serum via intrasplenic injection. Control groups consisted of tolerized neonatal rats that did not receive transplantation; tolerized rats with transplantation only; tolerized rats without transplantation plus HCV inoculation; non-tolerized rats without transplantation plus HCV inoculation; and tolerized rats, transplanted with Huh 7 cells and inoculated with HCV.

Visualization of transplanted Huh 7 cells, and HCV gene expression in rat livers

Immunohistochemical analyses for the presence of human albumin, and HCV viral protein in rat livers were done by a method similar to that published previously[6] with minor modifications. One mm sections of the middle portions of the left and median lobes were fixed for 24 hours in a 5% acetic acid-95% methanol mix following removal from animals. Five µm cryosections were cut from the fixed tissues, fixed in 100% acetone for 10 minutes, followed by incubation for 30 minutes in a 1:4 (30% hydrogen peroxide:methanol) solution at 25°C. Slides were washed extensively with water after fixation. Following quenching for 30 minutes with 1% BSA + 1% Tween-20 in PBS, pH 7.4 solution, slides were incubated in primary antibody from affinity purified rabbit anti-human albumin (Sigma, St Louis, MO) or rabbit anti-HCV NS5a protein

(Maine Biotechnology, Portland, ME) at 1/1,000 dilution, for 1 hour at 25°C. Following extensive washing with PBS, slides were exposed to 1/500 dilution of anti-rabbit rhodamine-labeled IgG (Jackson Labs) for 30 minutes at 25°C followed with PBS washes. Liver sections were visualized with a fluorescent microscope (Olympus BH 2), and digital images captured with an Olympus Camedia C-3040 zoom camera.

Detection of HCV RNA in rat serum

HCV (+) RNA was detected in serum using a 2-step nested PCR method. RNA was extracted from 10 µl rat serum using TRIzol LS (Invitrogen, Carlsbad, CA) according to manufacturer's specifications with minor modifications. Glycogen, 20 µg (Invitrogen), was added to the Trizol-serum mix before the homogenization step or at the time of RNA precipitation. The final RNA pellets were suspended in 10 µl RNase-free water for reverse transcription polymerase chain reaction (RT-PCR) reactions. Reverse transcription reactions were carried out using 5 U rTth polymerase (Perkin-Elmer, Boston, MA) with 30 pmole each of sense primer, nt 15-32: GGGGGCGACACTCCACCA, and antisense primer, nt 256-274: TCGCGACCCAACACTACTC, for 20 minutes at 65°C. Following inactivation of rTth polymerase activity, and addition of PCR reaction mixture, amplification was carried out using 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and the final elongation reaction at 72°C for 7 min. For the second nested-PCR step 1/50th of the final product of the first PCR reaction was used together with 2.5 U of AmpliTaq (Invitrogen) and 30 pmole of each primer, sense nt 35-55: AATCACTCCCCTGTGAGGAAC, antisense, nt 238-258: CTCGGCTAGCAGTCTTGCGG followed by similar cycling conditions as in the first step.

Twenty µl of the final PCR product was applied onto a 2% agarose gel to determine the size of final PCR product.

Detection of HCV replication in rat liver

HCV replication was detected by the presence of negative strand HCV RNA in rat liver using a combination of NTAG-reverse transcription PCR followed by nested PCR[7]. In brief, 1 µg of total liver RNA was reverse transcribed with 200 U SuperscriptII enzyme (Invitrogen), 2 pmole nTag-sense primer, nt 82-99: 5'-CCTCCGCGGCCGTCATGGTGGCGAATAAGCCATGGCGTTAGTATGAGTG-3' for 1 hour at 55°C. After inactivating Superscript II, PCR reactions were carried out with 200 U AmpliTaq Gold enzyme (Invitrogen) with 30 pmole of nTag-primer: 5'-CCTCCGCGGCCGTCATGGTGGCGAATAA-3'; 30 pmole of antisense primer, nt 256-274: 5'-TCGCGACCCAACACTACTC-3' and 40 cycles PCR reaction at 94 C for 1 min, 60 C for 30 sec, 72 C for 1 min. The second step nested PCR reaction was performed as mentioned above using sense primer: 5'-GAGTGTCGTGCAGCCTCCAG-3' and antisense primer: 5'-TACACCGGAATTGCCAGGAC-3'. The final PCR product was analyzed on 2% agarose and further confirmed by Southern blotting using HCV specific antisense probe: ^{32}P-TACACCGGAATTGCCAGGAC labeled with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]-ATP (Amersham, Piscataway, NJ).

Real time PCR quantitation of HCV RNA

RNA was extracted from 10 µl rat serum using TRIzol LS as described above. Reverse transcription was performed using iScript Reverse Transcriptase (Bio-Rad Labs, Hercules, CA) and iScript cDNA Synthesis kit (Bio-Rad Labs) with incubation at 25°C for 3 min followed by reverse transcription reaction at 42°C for 30 min, and inactivation of iScript RT enzyme at 85°C for 5 min. One tenth of product was used for real time PCR reaction using a iCycler iQ Real-time PCR Detection System (Bio-Rad Labs) with iQ SYBR Green Supermix and 50 nM each of forward primer (HCV 58-77 nt): 5'-CTGTCTTCACGCAGAAAGCG-3'; and reverse primer (HCV 313-294 nt) 5'-CACTCGCAAGCACCCTATCA-3'[8] with 1 cycle of 95°C for 5 min and 53 cycles of denaturation at 95°C for 20 sec followed by renaturation at 62°C for 1 min. Results were analyzed with iCycler iQ Real Time Detection Software 3.0 and quantitation expressed as means ± S.D. in units of copies/ml serum.

Serum aminotransferase assays

To detect possible liver damage following inoculation of HCV, aliquots of rat serum, 10 µl, were assayed spectrophotometrically for ALT enzyme activity using kits (Sigma) as instructed by the manufacturer. All assays were performed in triplicate and results expressed as means ± S.D. in units of IU/L.

Liver histology

Liver specimens were fixed in formalin, and stained with hematoxylin and eosin to evaluate liver architecture, and cellular reaction, and examined in a blinded fashion.

RESULTS

To detect HCV gene expression, samples were stained with an antibody against NS5A, a virally encoded protein that is required for the function of NS5B, the HCV RNA-dependent RNA polymerase. Fig. 1, panel 1C, shows that 14 weeks after HCV inoculation into rats tolerized and transplanted with human Huh 7 cells, groups of cells (6% of the total liver) stained positive for the presence of human albumin. In the same liver, panel 2C, groups of cells (1.9% of the total liver) were also positive for HCV NS5A antibody staining. The location of NS5A staining appeared to be cytoplasmic. In contrast, control rats that were untreated, panels 1A and 2A, failed to show staining for either human albumin or HCV NS5A indicating that the signals observed in panel 1C and 2C were not due to non-specific staining. Rats that were tolerized, not transplanted, but were inoculated with HCV, panels 1B and 2B, also had no NS5A staining demonstrating that the results in panel 2C were not due to an artifact of the HCV inoculation. Eighty-one percent (n = 57) of all tolerized, Huh 7 transplanted rodents had cells positive for human albumin in the livers. Sixty-two percent (n = 47) of all transplanted animals inoculated with HCV were positive for NS5A HCV protein by immunofluorescence studies.

Fig. 2 shows the results of RT-PCR for HCV (-) strand RNA using a nested PCR method that was 10,000 times more sensitive for HCV (-) strand compared to (+) strand, with 3,000 copies as the lower limit of sensitivity. Livers from rats tolerized, transplanted and inoculated with HCV, at 7 and 14 weeks, lanes 8 and 9; liver from a patient infected with HCV of the same genotype, lane 2; and control *in vitro*-generated HCV negative strand transcript, lane 3; all showed signals corresponding in migration to a size expected for the amplified sequence of 161 bp, compared to size markers in lane 1. There were variations in levels as the signal in one rat sample at 7 weeks,

lane 8, was visibly less than in another rat at 14 weeks, lane 9. Control animals that were only tolerized, lane 6, tolerized and transplanted, but not inoculated with HCV, lane 5, showed no signal under the same conditions. Rats that were tolerized, not transplanted, but inoculated with HCV, lane 7, showed a smeared signal, but no band corresponding to the expected position for a genuine HCV amplification product.

To determine whether the PCR product shown in Fig 2, lane 9, was truly amplified from an HCV sequence, Southern blotting of the amplification product was performed. Fig 3, lane 1 shows a representative blot from liver of a tolerized, transplanted and HCV inoculated rat at 14 weeks after inoculation. A band corresponding to 161 bp was found to hybridize with the HCV-specific probe in lane 1, as did the positive control from the liver of an HCV genotype 1-infected patient in lane 4. Untreated control rats, lane 2, as well as tolerized and HCV inoculated but not Huh7 transplanted, lane 3, failed to demonstrate any hybridization signal.

Real time PCR of HCV (+) strand RNA in serum was performed to quantitate HCV RNA levels as a function of time, and the results are shown in Fig. 4. In a group of tolerized, transplanted and inoculated rats (n=5), HCV RNA levels were measured at 11,000 copies/ml by week 4, rose to 20,000 copies/ml by week 8, reached a peak of approximately 22,500 copies/ml by week 12, and decreased to about 15,000 copies by week 16. In rats tolerized, not transplanted, but inoculated with HCV (n = 5), one animal had low levels (2,500 copies/ml) of HCV RNA at week 2, but had no detectable levels beyond week 8. The rest of the animals in this group were negative for HCV RNA through out the time course of study. The results indicate that the observed elevated HCV RNA levels beyond 8 weeks detected in transplanted-inoculated rats could not be due to residual

input HCV. Furthermore, rats that were tolerized and transplanted, but not injected with HCV were also negative, confirming that the observed results were not due to a non-specific amplification of nucleic acids from human cells in the rats (data not shown).

To determine whether HCV replication in the immunocompetent rats resulted in liver damage, ALT levels were assayed in serum as a function of time. Fig. 5 shows that while in control rats ALT levels remained persistently normal, in rats tolerized, transplanted, and inoculated with HCV, ALT levels became elevated to 60 IU/L by week 4, and reached a peak of about 120 IU/L by week 13. These levels remained elevated through at least through week 14.

Figure 6, panel A, shows that in livers from tolerized, transplanted and HCV-inoculated rats, focal collections of mononuclear inflammatory cells and Kupffer cells were found scattered in portal and central regions of the lobule. In contrast, rats tolerized and transplanted, but not injected with HCV, panel C, had no evidence of inflammation. The observed inflammatory response could not have been due to tolerization or transplantation of human cells alone. Similarly, non-tolerized, non-transplanted rats inoculated with HCV, panel B, also failed to show any inflammatory response confirming that HCV inoculation alone did not result in any histological evidence of inflammation.

DISCUSSION

Despite considerable efforts, the development of model systems to study HCV has been difficult. Direct infection of primary cultures with HCV has been described with chimpanzee hepatocytes[9], tree shrew (*Tupaia*) hepatocytes[10], and with human liver cells.[11] Because of the

difficulty in obtaining uniformly viable, and functional primary cultured hepatocytes, several investigators have studied the possibility of using well differentiated human hepatoma cell lines as a model for HCV infection.[12] However, these models resulted in only short term viral gene expression. More recently, subgenomic[13] and genomic[14,15] HCV RNA replicons have been developed. Mouse models have been developed recently including transfection of HCV RNA into mouse livers for transient expression.[16] Using a system more closely related to natural infection, Mercer *et al.*[17] developed a chimeric human liver in which human hepatocytes were transplanted into SCID-uPA mice. These animals were subsequently infected with HCV by inoculation. Our model differs from this one in several respects. Because of the SCID background required to prevent rejection of the human transplants, these animals have no immune response to viral antigens.[18] Because the inflammatory response is considered to be the major mechanism of liver damage during HCV infection[19], the presence of a normal immune system could theoretically reflect the natural immune response in man. In addition, the rats described in the current work do not require special germ-free environments, are not abnormally susceptible to bacterial infection, and therefore, are easier to maintain. There is also no genetic element present to induce elimination of host rat hepatocytes. As a result, the animals have a much lower population of human cells than reported in the uPA model. This is likely a major explanation for the levels of circulating virus, a maximum of which was measured to be 22,500 copies/ml. While this is relatively low compared to viral loads in man that not infrequently exceed 2 million copies/ml, the data indicate that the numbers of human cells in this model is sufficient to enable infection and replication of the virus, with quantifiable levels of virus circulating in serum. Furthermore, the finding of elevation of ALT, and inflammatory infiltrates on histology suggest that presence of a normal immune system results in HCV-related liver

damage. In this respect, it is recognized that there exists a mismatch between the rat immune system and human MHC antigens on the transplanted Huh 7 cells.[20] Thus, classical presentation of HCV antigens, and recognition by rat immunocytes would not be expected to occur. However, it is possible that cytokine-mediated effects are involved in the generation of the observed inflammation. Such by-stander effects have been described previously in other species-mismatched situations.[21, 22] While the exact mechanism by which the liver damage occurs in the current model is not entirely clear, the immune system is involved as evidenced by the inflammatory infiltrates in the liver. The data presented is the first description of a model of HCV infection which results in molecular, biochemical, and histological evidence of infection and hepatitis in an immunocompetent laboratory rodent. The model may be of value in studying the details of HCV infection, receptors and intracellular host factors involved, as well as in screening antiviral agents. With regard to the latter, the possible utility in testing new immunomodulatory agents and vaccines represents a unique advantage.

REFERENCES

1. Feinstone SM, Alter HJ, Dienes HP, Shimizu Y, Popper H, Blackmore D, Sly D, London WT, Purcell RH. Non-A, non-B hepatitis in chimpanzees and marmosets. J Infect Dis 1981;144(6):588-598.
2. Jarvis LM, Ludlam CA, Ellender JA, Nemes L, Field SP, Song E, Chuansumrit A, Preston FE, Simmonds P. Investigation of the relative infectivity and pathogenicity of different HCV genotypes in hemophiliacs. Blood 1996;87(7):3007-3100.
3. Yuh, DD, Gandy KS, Hoyt G, Reitz BA, Robbins RC. A rodent model of in utero chimeric tolerance induction. J Heart Lung Transp 1997;16(2):222-230.

4. Ouyang, EC, Wu, CH, Walton, CM, Promrat, K, Wu, GY. Transplantation of human hepatocytes into tolerized genetically immunocompetent rats. Wld J Gastro 2001; 7:324-330.

5. Wu CH, Ouyang EC, Walton CM, Wu GY. Human hepatocytes transplanted into genetically immunocompetent rats are susceptible to infection by hepatitis B virus in situ. J Viral Hepatitis 2001;8:111-119.

6. Osborne M, Weber K. Immnofluorescence and immunocytochemical procedures with affinity purified antibodies:tubulin-containing structures. Meth Cell Biol 1982;24:97-132.

7. Mizutani T, Ikeda M, Saito S, Sugiyama K, Shimotohno K, Kato N. Detection of negative-stranded hepatitis C virus RNA using a novel strand-specific reverse transcription-polymerase chain reaction. Virus Res 1998;53:209-214.

8. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M. Real time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116(3):636-642.

9. Lanford RE, Sureau C, Jacob JR, White R, Fuerst TR. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 1994;202:606-614.

10. Zhao, X, Tang, Z-Y, Klumpp, B, Wolff-Vorbeck, G, Barth, H, Levy, S, von Weisacker, F, Blum, H, and Baumert, TF. Primary hepatocytes of *Tupaia belangeri* as a potential model for hepatitis C infection. J Clin Invest 2001;109:221- 232.

11. Fournier, C, Sureau, C, Coste, J, Ducos, J, Pageaux, G, Larrey, D, Domergue, J, and Maurel, P. In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. J Gen Virol 1998;79: 2367-2374.

12. Song, ZQ, Min, F, Ma, QY, and Liu, GD. Hepatitis C virus infection of human hepatoma cell line 7721 *in vitro*. Wld. J Gastroenterol 2001;7(5):685-689.

13. Lohmann, V, Korner, F, Koch, J-O, Theilmann, L, and Bartenschlager, R. Replication of subgenomic hepatitis C virus RNA in a human hepatoma cell line. Science 1999;285:110-113.

14. Blight,KJ, McKeating, JA, and Rice, CM. Highly permissive cell lines for subgenomic, and genomic hepatitis C viral replication. J Virol 2002;76:13001-13014.

15. Ikeda, M, Yi, M, Li, K, and lemon, SM. Selectable subgenomic and genome length dicistronic RNAs derived from an infectious clones of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol 2002;76:2997-3006.

16. McCaffrey, AP, Meuse, L, Karimi, M, Contag, CH, and Kay, MA. A potent and specific morpholino antisense inhibitor of hepatitis C translation in mice. Hepatol 2003;38:503-508.

17. Mercer DF, Schiller DE, Elliot JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JRT, Tyrrell DLJ and Kneteman N. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7(8):927-933.

18. Brown JJ, Parashar B, Moshage H, Tanaka KE, Engelhardt D, Rabbani E, Roy-Chowdhury N, Roy-Chowdhury J. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. Hepatology 2000;31(1):173-181.

19. Perillo RP. Acute flares in chronic hepatitis B: the natural and unnatural history of an immunologically mediated liver disease. Gastroenterology 2001;120:1009-1022.

20. Lafferty KJ, Prowse SJ, Simeonovic CJ, Warren HS. Immunobiology of tissue transplantation: a return to the passenger leukocytes concepts. Ann Rev Immunol 1983;1:143-173.

21. Wang H, DeVries ME, Deng S, Khandaker MH, Pickering G, Chow LH, Garcia B, Kelvin DJ, Zhong R. The axis of interleukin 12 and gamma interferon regulates acute vascular xenogeneic rejection. Nature Medicine 2000;6:549-555.

22. Doherty PC, Christensen JP. Assessing: The dynamics of virus specific T cell responses. Ann Rev Immunol 2000;18:561-592.

LEGENDS

Figure 1. Localization of human albumin and HCV NS5a proteins in livers of HCV inoculated rats tolerized and transplanted with Huh 7 cells at 14 weeks. Human liver cells and HCV positive cells were localized using anti-human albumin (panels 1A, 1B and 1C) and anti-NS5a antibodies, respectively (panels 2A, 2B and 2C) and visualized by rhodamine-labeled secondary antibodies. Panels 1A and B, a representative liver from control, untreated rats; panels 2A and B, a representative liver from a control rat tolerized to Huh 7 cells, and inoculated with HCV; and panels 3A and B, rats tolerized, transplanted and inoculated with HCV.

Figure 2. Results of a combination nTag-PCR and nested PCR method for HCV negative strand assay to detect HCV replication in rat livers using HCV-specific primers. HCV-infected human liver (lane 2), *in vitro*-generated HCV-negative strand transcript control (lane 3), livers from tolerized rats, transplanted with Huh7 cells and inoculated with HCV at 7 weeks (lane 8), and 14

weeks (lane 9), liver of a tolerized only rat (lane 6), liver from a rat tolerized and transplanted with Huh 7 cells but not inoculated with HCV (lane 5), or from a rat tolerized, not transplanted but inoculated with HCV (lane 7). Lane 4 is a PCR control using buffer blank from the first PCR step.

Figure 3. Southern blot of 161 bp amplification products from two step PCR reactions for HCV negative strand. The amplified products were probed with an end-labeled HCV-specific antisense probe, and separated on a 2% agarose gel. A representative sample from a rat tolerized, transplanted, and inoculated with HCV at 14 weeks after infection (lane 1), a positive control liver of a human HCV-infected patient (lane 4), untreated control rat (lane 2) and liver from a representative rat tolerized and HCV-inoculated but not transplanted with Huh 7 cells (lane 3).

Figure 4. Time course of HCV RNA-positive in rats by real time PCR. RNA was extracted, reverse transcribed, and amplified as described in Materials and Methods. Data from rats tolerized, transplanted, and inoculated (n=5), squares; from rats tolerized only and inoculated with HCV (n=5), circles. All assays were performed in triplicate, and results expressed as means $\pm$ S.D. in units of copies/ml. The asterixes and crosses indicates values that were statistically significant between the two groups at a given time point.

Figure 5. Serum alanine aminotransferase (ALT) levels as a function of time. Aliquots of rat serum were assayed spectrophotometrically for ALT enzyme activity as described in Materials and Methods. Rats tolerized, transplanted and inoculated with HCV, squares; tolerized not transplanted, but inoculated with HCV, diamonds; tolerized and transplanted, but not inoculated

with HCV, circles; untreated controls, triangles. All assays were performed in triplicate and results expressed as means ± S.D. in units of IU/L.

Figure 6. Hematoxylin and eosin staining of rat liver sections at week 14. Panel A, a representative liver section from a tolerized, transplanted and HCV-infected rat; Panel B, a liver section from a tolerized rat without transplantation, but inoculated with HCV; Panel C, a liver section from a tolerized and transplanted rat, without HCV inoculation. Magnification 40X.

Presented, in part, at the annual meeting of the American Association for the Study of Liver Disease, October 2003

FIGURES

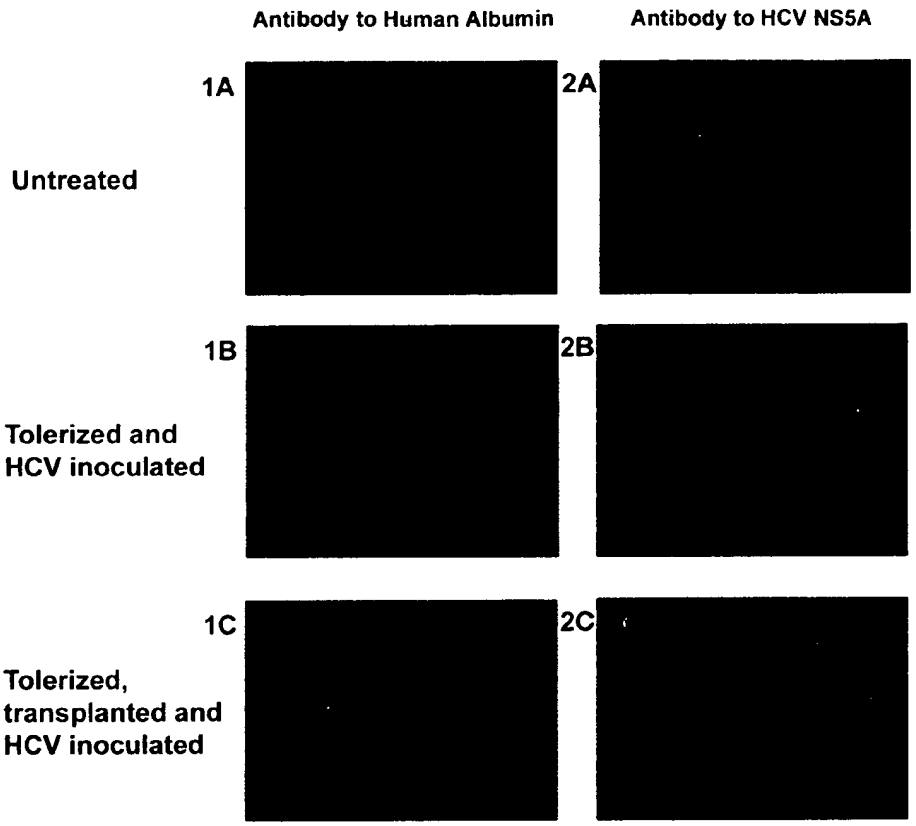

Figure 1

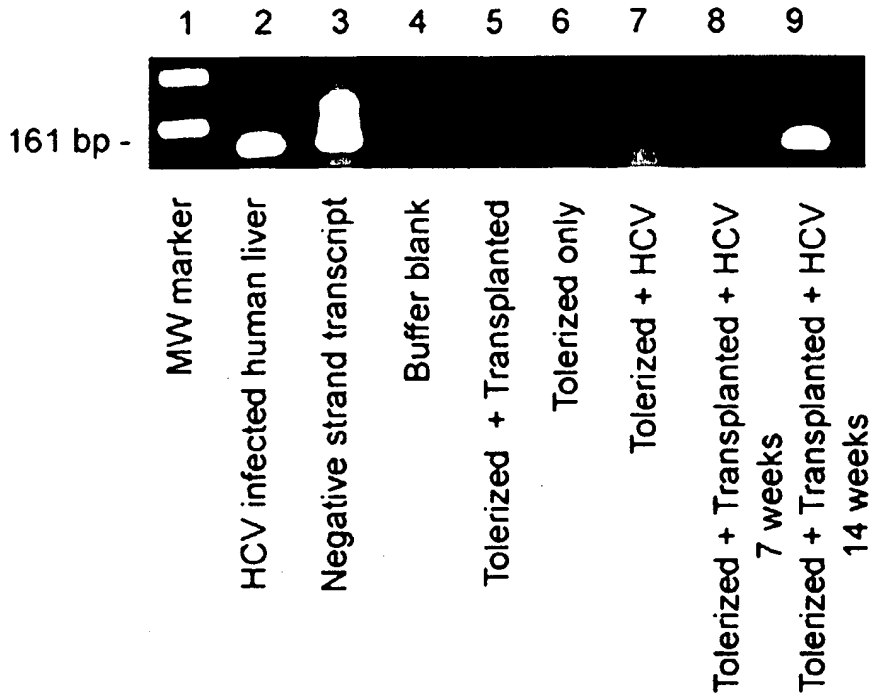

Figure 2

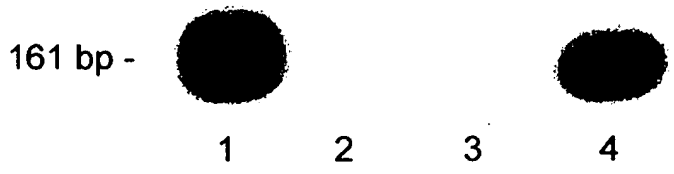

Figure 3

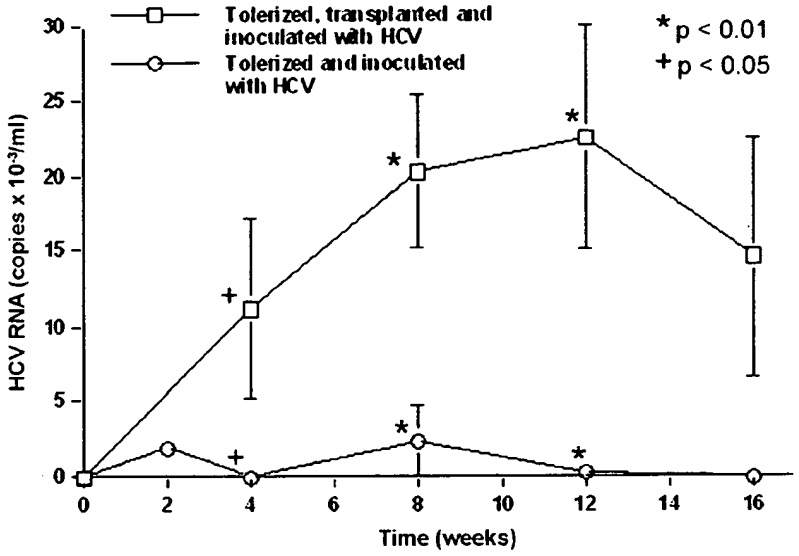

Figure 4

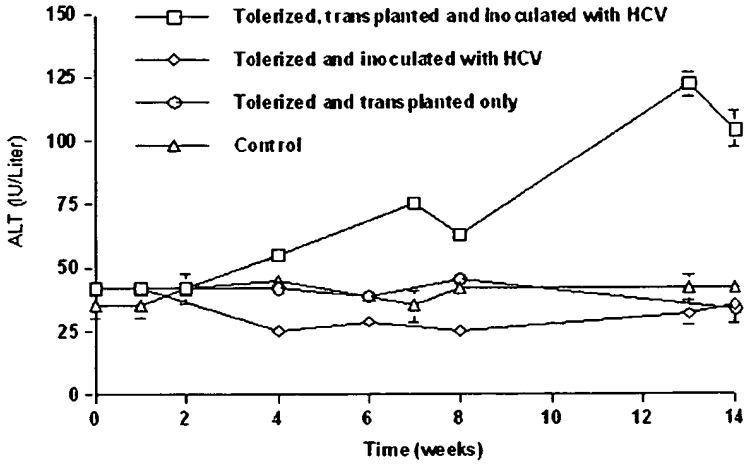

Figure 5

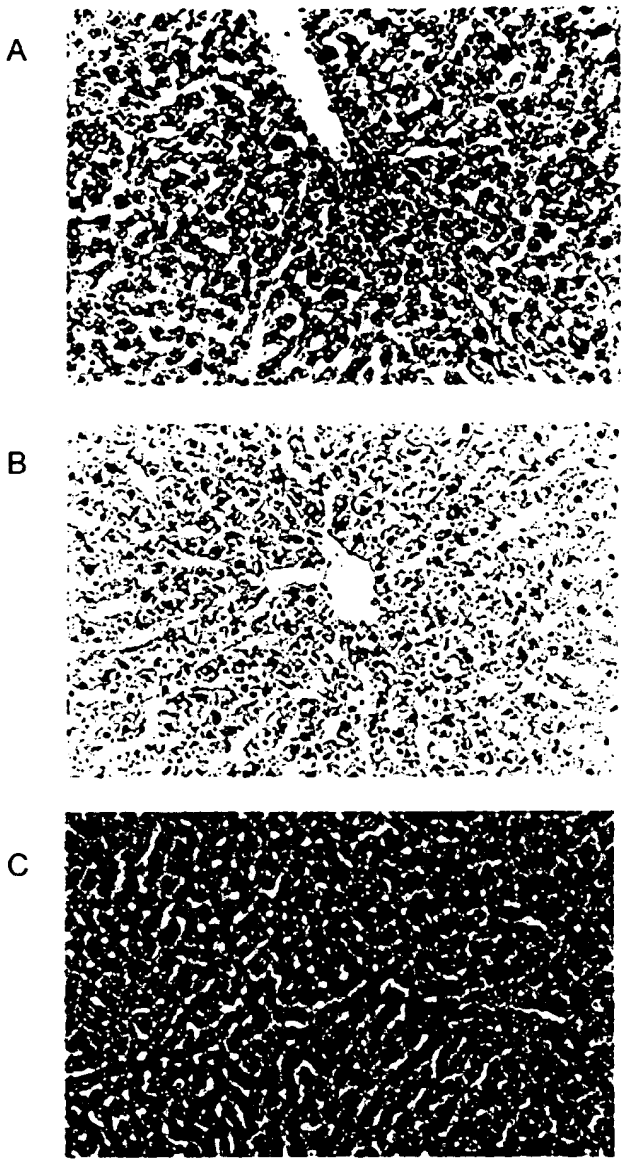

Figure 6